AMINO ACID SUPPLEMENTATION OF PROTEINS AND PROTEIN HYDROLYSATES

HENRY S. WYZAN, CHARLES F. KADE, JR.[1]
AND JESSY R. SHEPHERD
Biology Division, Sterling-Winthrop Research Institute, Rensselaer, New York

FOUR FIGURES

(Received for publication January 3, 1950)

It is well established (Rose, '38) that rats will grow on diets wherein amino acids provide practically the sole source of nitrogen. Several workers have demonstrated that the addition of sulfur-containing amino acids to casein diets results in better nitrogen utilization. Previous work in this laboratory has shown that the addition of 0.5% or 1.0% methionine to diets containing casein or various hydrolysates of casein increases the rate of growth and the protein efficiency. However, supplementation with 1.5% or more methionine is definitely inhibitory (Kade and Shepherd, '48). As little as 1% of methionine is sufficient to decrease the growth rate on 6% lactalbumin diets. A similar effect has been observed by Hier et al. ('44) with diets containing purified amino acids wherein a large proportion of nitrogen was furnished as glycine. Experiments in this laboratory indicate that excess glycine added to an 8% casein diet inhibits growth; that this inhibition is not dependent on the particular amino acid pattern of casein has been shown by using diets containing 6% lactalbumin.

During the above experiments on rats, determinations of the minimum nitrogen requirements of dogs were in progress. Since the minimum requirement for casein can be substanti-

[1] Present address: McNeil Laboratories, Inc., Philadelphia, Pa.

Copyright, 1950
The Wistar Institute of Anatomy and Biology
All rights reserved

ally reduced by supplementing the diet with a small amount of methionine, it seemed appropriate to determine the effect of several amino acids on nitrogen balance in the dog. This paper reports the results of feeding varying quantities of choline chloride and DL-methionine, L-cystine, L-cysteine, DL-alanine, glycine, DL-leucine, DL-phenylalanine, and L-tyrosine.

EXPERIMENTAL PROCEDURE

The procedure and diet employed were the same as those reported previously (Kade et al., '46, '48). The supplements of protein hydrolysates and amino acids were incorporated in the diet or fed separately by stomach tube before the nitrogen-free diet. All amounts fed were based on an optimum body weight represented by a value of the Cowgill-Drabkin "nutritive index" of 0.3 (Cowgill and Drabkin, '27).[2]

For the most part, the dogs employed in this investigation had been used in previous nutritional tests with satisfactory results. Usually they were brought to a state of nitrogen equilibrium by feeding diets containing minimum levels of casein or lactalbumin for several weeks, a procedure which has proved satisfactory in reducing carry-over effects between experiments.

All food and supplements were analyzed for nitrogen by the macro-Kjeldahl procedure, using copper sulfate as a catalyst. Except when the animals were fed intravenously, the dogs were catheterized only three days each week to give pooled specimens for two two-day and one three-day periods. During intravenous feeding, samples were collected and ana-

[2] This index is the ratio of the cube root of the body weight in grams to the body length in centimeters. Cowgill and Drabkin ('27) reported that in feeding experiments with dogs allowed to eat ad libitum, the animals voluntarily put themselves into a state of nutrition represented by a value of 0.3 for this ratio or "nutritive index." For reasons set forth in his paper, Cowgill ('28) considered this value to indicate an optimum state of nutrition voluntarily arrived at by the dog itself. Values significantly below this (0.28 and lower, for example) were believed to indicate states of nutrition below this optimum. By having all of our dogs show the value of 0.3, we really compared them at as nearly the same nutritive state as possible.

lyzed daily. Since no significant trends were seen, only the average weekly balances are reported. Feces were collected daily and pooled into 7-day specimens for nitrogen analysis.

RESULTS AND DISCUSSION

Figure 1 (a and b) illustrates the typical effects of supplementing hydrolysates with increasing amounts of methionine. Similar results were obtained with other dogs but are omitted to conserve space. These experiments demonstrate that the addition of up to 40 mg of nitrogen per kilogram of body weight (hereafter abbreviated to mg N/kg) as methionine enhances nitrogen retention. Doubling the methionine supplementation, however, not only threw the dogs drastically and suddenly out of balance but quickly caused deterioration of their physical condition. All dogs developed a syndrome of negative nitrogen balance, vomiting, loss of hair, the appearance of open skin lesions — particularly on the legs — and dry eyes hypersensitive to light. The apparent toxicity of methionine at these higher levels is severe enough to threaten death to the dog. It is difficult to portray in words the suddenness and severity of the change caused by the methionine. Only by extreme measures, including constant care and intravenous feeding, were these animals saved. In later experiments, the condition was recognized immediately and reversed in about 24 hours by simply omitting the methionine supplement. It is apparent that the animal receiving these high methionine supplements is unable to utilize amino acids properly, even though they are fed in amounts which are ample for normal needs.

A typical graph of the similar effects observed on 4 dogs fed lactalbumin and casein diets supplemented with methionine is shown in figure 1 (c). As is illustrated in this figure, the addition of 70 to 80 mg N/kg as methionine to a diet providing 150 mg N/kg as casein decreased utilization. Doubling the casein intake to 300 mg N/kg permitted adding up to 100 mg N/kg as methionine without visible ill effects, although nitrogen retention steadily declined to the level obtained with

unsupplemented casein. The addition of less than 40 to 50 mg N /kg as methionine to a diet furnishing 90 mg N/kg as lactalbumin did not significantly affect nitrogen retention. When 40 mg N/kg as methionine were supplied, nitrogen balance was not as well maintained as with lactalbumin alone. Toxic symptoms were delayed and less marked than those occurring with hydrolysates supplemented by methionine. It seems certain, however, that to continue at this level would have brought about serious physical deterioration.

Kade and his associates ('48) showed that 90 mg N/kg as lactalbumin or 140 mg N/kg as casein will maintain adult dogs in nitrogen balance. However, 90 mg N/kg as casein supplemented with 5 mg N/kg as methionine are adequate. The superior efficiency of lactalbumin in maintaining nitrogen balance is due to the fact that 90 mg N/kg furnish approximately as much of the sulfur-containing amino acids as do 140 mg N/kg of casein. Upon supplementing lactalbumin (90 mg N/kg) with 40 mg N/kg as methionine, the ratio of sulfur-containing amino acids to the total amino acids becomes approximately the same as that resulting from adding 80 mg N/kg as methionine to casein (150 mg N/kg). More methionine is necessary to bring about adverse symptoms when greater quantities of other amino acids are supplied, as is shown by the results obtained on the higher casein diets. At the 300-mg casein nitrogen intake sufficient other amino

Fig. 1 (a) Typical example of nitrogen balance of a dog fed an orally administered casein hydrolysate supplemented with increasing amounts of DL-methionine. The animal received hydrolysate 3679-S at 110 mg N/kg for the first three weeks and then at 90 mg N/kg for the last two weeks. The changes in level are indicated by the vertical lines.

(b) This dog was put on a regimen similar to (a) in which hydrolysate 1131-T was fed at 110 mg N/kg for the first three weeks and at 100 mg N/kg for the remainder of the experiment.

In both examples the last week was marked (see arrow) by the sudden onset of vomiting, weakness, loss of hair, open lesions, and hypersensitivity to light.

(c) Typical example of nitrogen balance of a dog as affected by methionine supplementation of a diet furnishing 90 mg N/kg as lactalbumin. The toxic symptoms noted in (a) and (b) were observed during the weeks marked by the arrows.

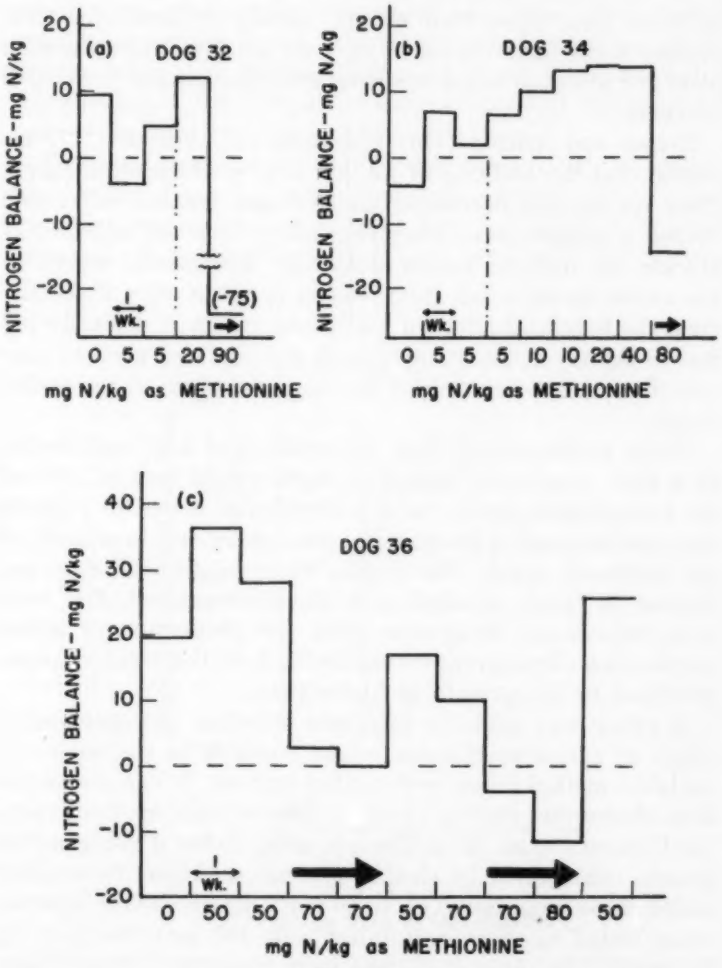

(a)
DOG 32
NITROGEN BALANCE - mg N/kg
20
10
0
-10
-20
1 Wk.
(-75)
0 5 5 20 90
mg N/kg as METHIONINE
(b)
DOG 34
NITROGEN BALANCE - mg N/kg
20
10
0
-10
-20
1 Wk.
0 5 5 5 10 10 20 40 80
mg N/kg as METHIONINE
(c)
DOG 36
NITROGEN BALANCE - mg N/kg
40
30
20
10
0
-10
-20
1 Wk.
0 50 50 70 70 50 70 70 80 50
mg N/kg as METHIONINE

Figure 1

acids are furnished so that 100 mg methionine nitrogen represent less than 50% of the total amino acids and are not sufficient to produce toxic effects. Levels of methionine furnishing more than 100 mg N/kg were not tried because such diets are prone to cause vomiting even though fed in divided portions.

Brown and Allison ('48) and Roth and Allison ('49) reported that the addition of an excess of methionine to casein diets fed to rats decreased the nitrogen balance index and caused a weight loss. They also noted that the addition of glycine or arginine counteracted the detrimental effect of the excess methionine. Since casein contains very little glycine, the beneficial effect of additional casein is probably not due to the glycine content, though the arginine present may contribute necessary groups for the metabolism of the methionine.

These workers noted that the addition of 4.8% methionine to a 12% casein diet caused a rapid weight loss in rats of the Long-Evans strain, but 7% methionine added to a casein diet was required to produce the same symptoms in animals of the Sherman strain. The results we obtained with dogs are similar to those obtained with the Sherman rat, for toxic symptoms began to appear when the proportion of added methionine nitrogen was roughly half of the total nitrogen provided by the protein or hydrolysate.

A study was made to determine whether the detrimental effect of excess methionine might possibly be due either to its labile methyl group or its sulfur content. It has previously been shown that choline also can furnish labile methyl groups (duVigneaud et al., '41). Thus, in order to see if labile methyl groups contributed by choline produce a similar toxic effect under these conditions, up to 80 mg N/kg as choline chloride were added to diets containing both 150 and 300 mg N/kg as casein. No toxic symptoms were observed. Typical data obtained at the 150 mg N/kg casein level are illustrated in figure 2. Low levels of choline chloride supplementation had no effect on nitrogen balance, whereas the addition of small

amounts of methionine to casein diets caused increased nitrogen retention. This result was not unexpected, since the basal diets presumably contained adequate labile methyl groups. Inasmuch as the animals remained in normal physical condition even though as high as 80 mg N/kg as choline were added to the diet, an excess of potential labile methyl groups was not the cause of toxicity.

To investigate the possibility that the toxicity of methionine arises from its sulfur-containing moiety, cystine and cysteine were used to supplement diets furnishing both levels of casein. Since the effects on all dogs fed cystine and cysteine

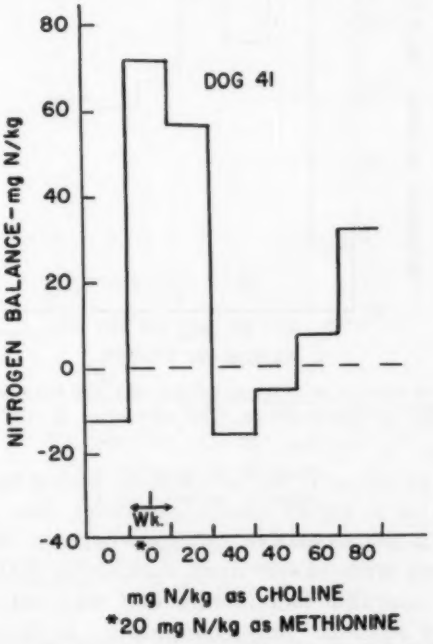


Fig. 2 Typical example of nitrogen balance of a dog as influenced by choline chloride supplementation of a diet furnishing 150 mg N/kg as casein. During the second week of this experiment, 20 mg N/kg as methionine were given. A carrying-over or prolongation of the methionine effect followed by decreased retention for one week is noticeable here. This is not unusual.

are similar, and in order to conserve space, the data on only one animal are shown in figure 3. The addition of 20 mg N/kg as cystine had a beneficial effect on nitrogen balance; increasing the amount to 150 mg N/kg and 300 mg N/kg, respectively, brought about neither increased retention nor adverse physical symptoms. Because of its low solubility, a large proportion of the cystine fed was not absorbed but was eliminated in the feces.

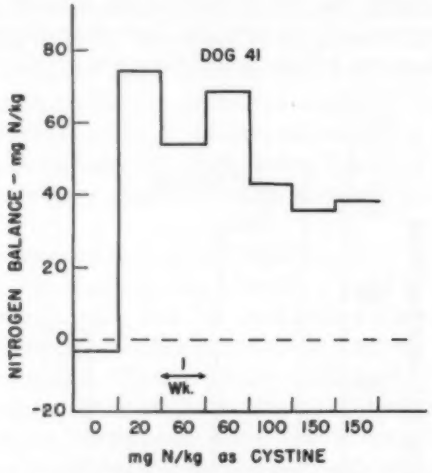


Fig. 3 Typical example of nitrogen balance of a dog fed cysteine- or cystine-supplemented diets providing 150 mg N/kg as casein.

As much as 50 mg N/kg as cysteine added to a diet furnishing 150 mg N/kg as casein produced, like cystine and methionine, a high positive nitrogen balance. Similar data were obtained with casein diets furnishing 300 mg N/kg as casein. An adequate lactalbumin diet was not significantly improved by cysteine supplementation. It is necessary to add more nitrogen (approximately 10 mg N/kg) as cysteine than as methionine (5 mg N/kg) to obtain nitrogen balance when 90 mg N/kg as casein are fed. Like methionine, cysteine is well absorbed and causes vomiting at higher intake

levels. However, the symptoms of acute toxicity, so marked at high levels of methionine supplementation, did not appear even after prolonged feeding.

The biological value of any protein or mixture of amino acids will be largely dependent on the amino acid present in

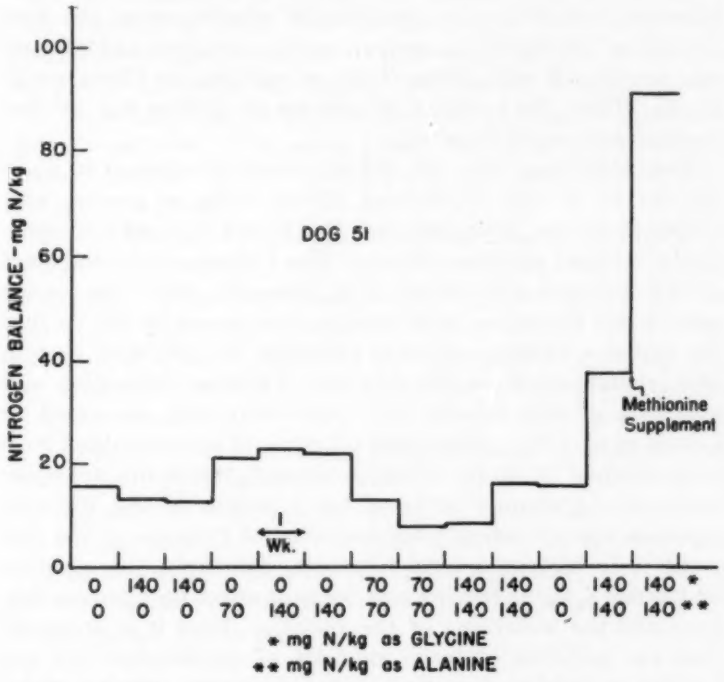


Fig. 4 Typical example of nitrogen balance of a dog fed glycine- and alanine-supplemented casein diets at 140 mg N/kg. Twenty milligrams N per kilogram as methionine were added during the last week of the test.

an amount which is lowest in proportion to its quantitative requirement. Methionine is the limiting amino acid in casein and its hydrolysates. The addition of amino acids other than methionine, therefore, should not augment nitrogen utilization. As is illustrated by a typical example in figure 4, the

biological value of casein, lactalbumin, and acid hydrolysates of casein, fed orally or intravenously and in quantities sufficient to maintain a dog in nitrogen equilibrium, was not enhanced by the inclusion of large amounts of glycine or alanine or both. Large quantities of these amino acids were not toxic and the animals remained in nitrogen equilibrium. Nitrogen retention was significantly greater when the diet providing 140 mg N/kg each as casein, alanine, and glycine was reinforced with 20 mg N/kg as methionine (final week, fig. 4). Thus, the addition of alanine or glycine has neither positive nor negative effect.

Two adult dogs (No. 56 and 64) were maintained in equilibrium on a diet furnishing 150 mg N/kg as casein plus 150 mg N/kg as phenylalanine for 5 and 6 weeks, respectively, without apparent illness. The animals converted part of the ingested phenylalanine to tyrosine. This was easily seen in dog 64, whose urine volume was generally low so that the tyrosine output regularly exceeded its solubility therein and crystallized from the solution. Tyrosine formation and excretion in male infants have previously been described by Levine et al. ('43). Also, dogs maintained on these diets with a supplement of up to 150 mg N/kg as tyrosine did not show toxicity. Apparently little of the nitrogen of the ingested tyrosine was absorbed, since inclusion of tyrosine in the diet resulted in increased fecal nitrogen. Similarly, the addition of 150 mg N/kg as leucine was without effect on nitrogen balance and the well-being of the animals. Thus it is apparent that the addition of large amounts of methionine, but not similar quantities of cystine, cysteine, alanine, glycine, phenylalanine, tyrosine, leucine, and choline, produces toxic symptoms and negative nitrogen balance in dogs.

SUMMARY

1. The addition of up to 40 mg N/kg as methionine to acid hydrolysates of casein given orally at maintenance levels in adult dogs increases nitrogen retention. Negative nitrogen balance and serious physical deterioration develop quickly

upon increasing the level of methionine supplementation above 40 mg N/kg.

2. The addition of up to 40 mg N/kg as methionine to a minimum lactalbumin diet or of up to 70 mg N/kg to a minimum casein diet has little effect on nitrogen retention. In both cases additional methionine causes toxic symptoms.

3. In general, under these conditions, the addition of more than 50% of the total amino acids as methionine creates an imbalance and seriously impairs utilization. It appears that the toxic effect of methionine-induced imbalance cannot be directly ascribed to its labile methyl group or sulfur content. Excess choline chloride, cystine, and cysteine were fed without adverse effect.

4. Glycine and alanine may be added to casein, to hydrolysates of casein, and to lactalbumin as complementary sources of nitrogen. These amino acids added individually or together have no influence on nitrogen retention. When the limiting amino acid of the protein or mixture is also furnished (as, for example, methionine to a casein diet), nitrogen retention is much increased.

5. Large amounts of phenylalanine added to a casein diet are apparently metabolized without difficulty. The tyrosine formed may be voided in the urine in quantities sufficient to exceed its solubility therein. The addition of tyrosine and leucine is without effect on nitrogen utilization. Tyrosine is poorly absorbed and at high intake levels a large proportion is eliminated in the feces.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Dr. Lloyd C. Miller in the preparation of this paper. Thanks are due to Mr. Calvin Zippin for preparation of the figures and to Mr. Daniel J. Bradley, Jr., for technical assistance.

LITERATURE CITED

BROWN, J. H., AND J. B. ALLISON 1948 Effects of excess dietary DL-methionine and/or L-arginine on rats. Proc. Soc. Exp. Biol. Med., *69:* 196.

COWGILL, G. R. 1928 The energy factor in relation to food intake; experiments on the dog. Am. J. Physiol., *85:* 45.

COWGILL, G. R., AND R. L. DRABKIN 1927 Determination of a formula for the surface area of the dog together with a consideration of formulae available for other species. Ibid., *81:* 36.

DUVIGNEAUD, V., M. COHN, J. P. CHANDLER, J. R. SCHENK AND S. SIMMONDS 1941 The utilization of the methyl group of methionine in the biological synthesis of choline and creatine. J. Biol. Chem., *140:* 625.

HIER, S. W., C. E. GRAHAM AND D. KLEIN 1944 Inhibitory effects of certain amino acids on the growth of young male rats. Proc. Soc. Exp. Biol. Med., *56:* 187.

KADE, C. F., J. HOUSTON, K. KRAUEL AND M. SAHYUN 1946 The maintenance of nitrogen equilibrium in dogs by intravenous alimentation with an acid hydrolysate of casein fortified with tryptophane. J. Biol. Chem., *163:* 185.

KADE, C. F., J. H. PHILLIPS AND W. A. PHILLIPS 1948 The determination of the minimum nitrogen requirement of the adult dog for maintenance of nitrogen balance. J. Nutrition, *36:* 109.

KADE, C. F., AND J. R. SHEPHERD 1948 The inhibitory effect of excess methionine on protein utilization. Fed. Proc., *7:* 291.

LEVINE, S. Z., M. DANN AND E. MARPLES 1943 A defect in the metabolism of tyrosine and phenylalanine in premature infants. III. Demonstration of the irreversible conversion of phenylalanine to tyrosine in the human organism. J. Clin. Invest., *22:* 551.

ROSE, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev., *18:* 109.

ROTH, J. S., AND J. B. ALLISON 1949 The effect of feeding excess glycine, L-arginine, and DL-methionine to rats on a casein diet. Proc. Soc. Exp. Biol. Med., *70:* 327.

MANGANESE DEFICIENCY IN RATS WITH RELATION TO ATAXIA AND LOSS OF EQUILIBRIUM [1]

ROBERT M. HILL, DORSEY E. HOLTKAMP, A. R. BUCHANAN AND ENID K. RUTLEDGE

Departments of Biochemistry, Anatomy, and Pathology, University of Colorado Medical Center, Denver

TWO FIGURES

(Received for publication February 9, 1950)

Caskey and Norris ('40) observed ataxia in the chicks of hens on a manganese-deficient diet. Shils and McCollum ('43) reported ataxia, incoordination, and poor equilibrium in manganese-deficient rats. These rats were hyper-active and exhibited "rotational movements" and "loss of the righting reaction." "Occasionally they walked in a straight forward manner, but soon fell over on the side or back." The symptoms increased in severity up to the 18th day of life, after which the rats either died or "recovered to a great extent."

In our laboratory we have succeeded in raising rats through several generations on a manganese-deficient diet. In animals placed on low manganese diets after maturity, no ataxic symptoms appeared. However, in the second generation, symptoms appeared in 5 females, two before 6 months and three between 6 and 14 months of age. The latter three of these females raised litters, although poorly. Of three males and 4 females, in the third generation, which were al-

[1] This study was financed in part by a grant from the Office of Naval Research. A preliminary report of this work, with motion pictures, was presented at the meeting of the Federation of American Societies for Experimental Biology in Detroit, April, 1949 (Hill, Holtkamp, Buchanan and Rutledge, '49).

lowed to reach maturity, one male and two females developed characteristic symptoms before weaning. One female of this generation that did not show symptoms successfully raised one of a litter of two. This 4th generation female rat exhibited marked ataxic symptoms and had reached 150 days of age when it was sacrificed for study.

In this paper we are reporting our observations on the relation of manganese intake to the development of ataxia and loss of equilibrium, as well as an attempt to locate the lesion responsible for these symptoms.

EXPERIMENTAL

The rats used in this study came originally from the Wistar strain. They have been inbred for many years at this institution. All the rats were kept in metal cages and were fed a milk diet which had manganese as the only variant. The average daily intake of an adult rat was 100 ml of milk, which was made up as follows: to 90 ml of distilled water were added 12.2 gm of spray-process dried, powdered, whole milk; 1.0 ml of corn oil;[2] 15 U.S.P. units of vitamin D;[3] 5 mg iron (as $FeSO_4$); 0.1 mg copper (as $CuSO_4$); 0.1 mg thiamine; 0.1 mg niacin; 0.005 mg folic acid;[4] and 18 mg *p*-aminobenzoic acid. In three rats of the first generation there were minor differences in the vitamin intake but not in the intake of manganese. The minerals were first added to the distilled water and the vitamins, oil, and powdered milk were then added. The entire mixture was completely homogenized in a Waring Blendor, and did not separate on standing.

One group of rats received this manganese-deficient diet without supplement. By analysis, the deficient diet contained an average of 0.03 mg manganese per 100 ml of milk. These analyses were carried out either by use of a modification of

[2] Mazola.

[3] Vitamin D_3, obtained commercially as Drisdol, Winthrop Chemical Co., New York, N. Y.

[4] Thiamine, niacin (as nicotinic acid amide), and folic acid were supplied by Lederle Laboratories, New York, N. Y., through the courtesy of Dr. Guy Clark.

the method of Ray ('40) or by a modification of the method of Kun ('47), which makes use of the catalytic properties of manganese. These two methods gave values in close agreement. Cows' milk without supplements has been assayed in other laboratories and shown to contain 0.001 mg to 0.006 mg manganese per 100 ml of milk (Broek and Wolff, '35; Ray, '40; Sherman, '46). We did not assay the powdered milk for manganese prior to the addition of the supplement. The differences in our assays on the fortified milk used and the assays reported in the literature presumably represent either manganese impurities in the dietary supplements or impurity added during the commercial processing of the milk.

For studies of other problems, 6 additional groups of animals were observed at the same time. These animals received the same diet except for the addition of a manganese supplement that ranged from 0.1 mg to 40.0 mg per adult rat per day. In none of the animals receiving a manganese supplement did the same symptoms of ataxia or loss of equilibrium develop. The further discussions of this paper are concerned primarily with the group to which no manganese supplement was given.

RESULTS

Sixteen animals of the "first generation," 8 females and 8 males, which were raised to breeding age in the stock colony, failed to show any symptoms of disturbance in equilibrium even as much as a year after having been placed on the manganese-deficient diet. Eight litters were produced from 6 females of this group. The average time the mothers were on the manganese-deficient diet before birth of the young was 45 days. One litter was lost at birth. In the other 7 litters, 58 young were born. Seventeen of these were sacrificed in order to reduce the litter size to a maximum of 6. Of the remaining 41, 34 were raised to 18 days of age. Of these 34, the "second generation," 8 females and three males were kept for breeding. The others were sacrificed for study. Five females of the second generation developed ataxia and loss of equilibrium, two before 6 months of age and the other

three before 14 months of age. The first two were sacrificed at 6 months of age for study; the other three were bred and produced young but reared only a few and those with difficulty. In the latter three females the symptoms of disturbance in equilibrium were observed only after one or two litters had been produced. This may have been due to loss of manganese to the fetuses, to loss during lactation, or to gradual loss during the long period of manganese deficiency. All these factors could, of course, be contributory.

Three males and 4 females of the third generation were allowed to reach maturity. Of this group, two females and one male showed definite symptoms and one male questionable symptoms of disturbance in equilibrium before 60 days of age. One third generation female that did not show characteristic symptoms successfully raised one of a litter of two. This one 4th generation female rat was sacrificed at 150 days of age. It was the only rat of the 4th generation of animals subjected to manganese deficiency that we were able to study.

The disturbance of equilibrium appeared at an earlier age with successive generations of manganese deficiency. Many animals of the third generation and the one 4th generation animal showed symptoms when they first began to walk, but few of these animals survived beyond 18 days of age.

A total of one adult male and 8 adult female rats on the manganese-deficient diet showed symptoms of ataxia and loss of equilibrium. In all the females the shoulders leaned toward the right and the head was rotated, with the lower jaw directed to the left and upward. Figure 1 A shows the typical position of an ataxic animal at rest. The one male had shoulders leaning to the left and head rotated with the lower jaw directed to the right and upward. The lesion, therefore, appears to be unilateral but can occur on either side. The leaning and rotational movements were always exaggerated if the animal was disturbed. If prodded, the severely affected animals would run in circles, frequently falling, and, on stopping, would invariably hyperextend the neck and, in extreme cases, fall over backward. Hyperextension of the

neck was the usual reaction to lowering the head to drink, which was similar to the drinking motions of chickens (fig. 1 B). When prodded, the ataxic rats frequently ran backward, a feat that was accomplished with better coordination than running forward.

The one 4th generation rat and three third generation rats on the manganese-deficient diet were compared to rats receiving 5 mg and 10 mg of manganese per day with regard to eye deviation and nystagmus following rotatory and caloric stimulation.

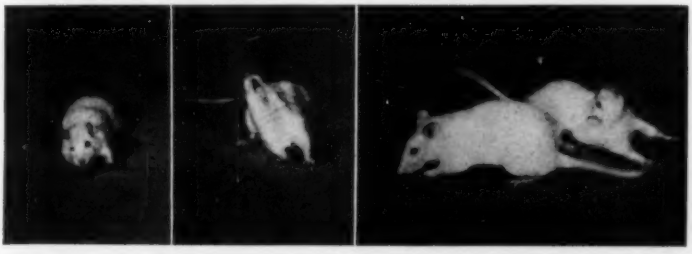


Fig. 1 A, ataxic rat showing typical position at rest; B, ataxic rat showing hyperextension of neck in response to stimulus; C, the normal rat (front) and the ataxic rat (rear) were rotated together in the same cage. Note the lack of response in the normal rat and the extreme hyperextension and rigidity of the ataxic rat.

The methods used were similar to those described by Buchanan ('37). The rats were held either in the ventral position or on the left side during rotatory stimulation. All the animals were in the ventral position during caloric stimulation. The use of caloric stimulation with water at 0°C. gave inconclusive results. The normal post-rotatory response was very definite, with nystagmus in the horizontal plane and directed opposite to the direction of rotation in both eyes. The manganese-deficient animals showed a nystagmus in the same direction as that found in the normal animals but slower, of greater amplitude and of shorter total duration. One of the third generation animals and a normal animal of the

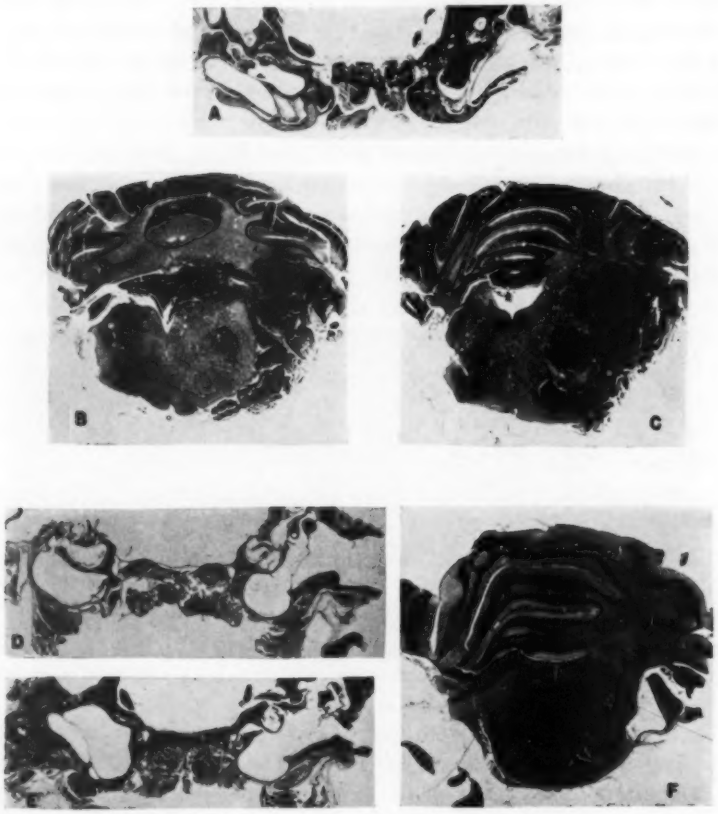


Fig. 2 A, temporal bones from a rat (not manganese-deficient) whose ataxic symptoms were due, at least in part, to otitis media and petrositis on the right; B, brain stem and cerebellum from the same animal as in A, showing extension from the temporal bone with development of a cerebellomedullary abscess; C, same as in B except at a more rostral level; D and E, temporal bones from a manganese-deficient rat (second generation) which was markedly ataxic, at two rostro-caudal levels showing normal external auditory canals, tympanic membranes, and tympanic cavities bilaterally; F, brain stem and cerebellum from the same animal as in D and E. No pathology was apparent upon either gross or microscopic examination. Magnifications: A, × 3.5; B, × 5.5; C, × 6; D, × 3.5; E, × 3.5; F, × 6. Stain: hematoxylin and eosin.

same age were rotated together in a wire cage, and immediately dumped on a table top and photographed. Figure 1 C shows the difference in response to this treatment. The normal animal, in the foreground, shows no ill effects. The response of the deficient rat is typical. Note the rigid tail that quivers enough to blur the picture.

The one 4th generation animal, three of the third generation and one of the second generation were sacrificed for study. The brains of 4 and the labyrinths of two of these rats were sectioned serially and alternate slides were stained with hematoxylin and eosin for cell studies; the intervening slides were stained for myelin by the method of Weil ('28). The carcasses minus the brains and gastrointestinal tracts of the one 4th generation rat and of two of the third generation rats were analyzed for manganese. A modification of the method of Ray ('40), in which potassium periodate is used to oxidize the manganese to permanganate instead of persulfate, was employed. The color readings were made on a Leitz colorimeter using a "D" filter of about 525 mμ maximum transmission.

The histological preparations were compared with those from rats which had received supplements of manganese and to those from animals with labyrinthitis caused by specific infection (fig. 2). Labyrinthitis, secondary to infection of the middle ear, develops not infrequently in manganese-deficient and other rats and accounts for ataxic symptoms. Corynebacterium pseudotuberculosis, which has been isolated from pulmonary lesions that occur occasionally in our rat colony, has been found also in a cerebellomedullary abscess, secondary to petrositis and labyrinthitis. Such an infection in a non-manganese-deficient rat is shown in figure 2 A, B, C, and involved the middle ear, petrosa, medulla and cerebellum. Ataxia, as severe as that due to such lesions, occurs in manganese-deficient animals without demonstrable lesions in hematoxylin and eosin- or Weil-stained sections of the temporal bone or brain (fig. 2 D, E, F). No demyelination in

the brain stem or 8th nerves was evident in the Weil-stained sections.

In table 1 the manganese analyses are compared with those made on rats that received manganese supplements and with analyses reported by Skinner, Peterson and Steenbock ('31).

TABLE 1

Manganese content of rats on different levels of dietary manganese

RAT NO.	GENERATION NO.	DIETARY MANGANESE INTAKE PER ADULT PER DAY	LIVE BODY WEIGHT	MANGANESE PER 100 GM LIVE BODY WEIGHT [1]	MANGANESE PER RAT	OTHER MANGANESE PER RAT DATA [2]		AVERAGE MANGANESE CONTENT OF CARCASS IN TERMS OF DAILY INTAKE
						Limits	Average	
		mg	*gm*	*mg/100 gm*	*mg*	*mg*	*mg*	*days*
1	IV	0.03	130	0.032	0.041			
2	III	0.03	164	0.018	0.029			1.1
3	III	0.03	164	0.020	0.033			
		0.1				0.050–0.085	0.068	0.68
4	IV	1.0	188	0.045	0.084			
5	IV	1.0	167	0.045	0.076			0.1
6	IV	1.0	170	0.080	0.137			
		5.0				0.088–0.149	0.113	0.02
7	IV	10.0	160	0.269	0.430			
8	IV	10.0	133	0.125	0.167			0.05
9	IV	10.0	144	0.652	0.904			
10	III	0.03 [3]	180	0.027	0.048			
11	III	0.03 [3]	186	0.014	0.027			1.2
12	III	0.03 [3]	222	0.014	0.032			

[1] Manganese analyses on carcasses minus brains, brain stems, and gastrointestinal tracts.

[2] Data of Skinner, Peterson and Steenbock ('31).

[3] These rats were of the third generation of a group of rats receiving 0.1 mg manganese per adult per day. They received this diet until weaning age, but received no manganese supplements thereafter. They were sacrificed at 250 days of age.

The data show the effect of the level of manganese in the diet on manganese storage.

Skinner et al. ('31) fed rats on "low" and "high" manganese diets and presented data on the manganese content of these animals on the 180th day of life. The "low" manganese diet contained about 0.1 mg manganese per rat per day, with corresponding carcass analyses averaging 0.0676 mg of total manganese and varying from 0.050 to 0.085 mg. The "high" manganese diet contained 1.1 mg of manganese per day from the age of weaning to 70 days and thereafter about 5.1 mg per rat per day. These rats showed an average carcass analysis on the 180th day of 0.1130 mg, with variations from 0.088 to 0.149 mg. The dietary manganese levels in these rats were in each case intermediate between the levels fed the groups in our experiments. The corresponding carcass analyses show manganese at comparable levels (table 1).

The last three rats listed in table 1 (numbers 10, 11 and 12) are from a group of 23 animals of the third generation receiving 0.1 mg manganese per rat per day. These 23 rats, at the time of weaning, were placed on the no-supplement diet. Although, when sacrificed at 250 days of age, the three rats showed carcass manganese levels in the same range as those of rats 1, 2, and 3, none of the 23 has shown symptoms of ataxia or loss of equilibrium at any time. The significance of this will be discussed.

DISCUSSION

A seemingly logical explanation of the ataxia and loss of equilibrium in our manganese-deficient rats would be that a lowered resistance resulting from the manganese deficiency had permitted the development of an infection of the middle ear. King ('39) describes the symptoms and the lesions of labyrinthitis in rats. Superficially, there is a similarity between the symptoms described by King and those of our animals, but there are certain discrepancies that suggest a fundamental difference. According to King, " . . . the rat holds its head continuously close to the floor of the cage,

with the nose pointing away from the median line." This was true of our animals when at complete rest. But even with the slight stimulus of their own voluntary movements, our animals, instead of holding the head close to the floor, raised it and pointed the nose upward and to one side. The stimulus of drinking or of prodding resulted in raising the nose to such a degree that the rat frequently fell over backward. King describes the inability to run in a straight line that we observed, but makes no mention of a strong tendency to run backward such as was present in our animals. King observed that, "Autopsies . . . disclosed extensive infection in the ear and lesions of the tympanic and petrous bones." In our animals no lesions could be discovered by histological examination, although hematoxylin and eosin- and Weil-stained serial sections were studied.

Rats 10, 11, and 12 of table 1 were on a diet of 0.1 mg manganese per day until the time of weaning, and only then were placed on the manganese-deficient diet. Nevertheless, the carcasses of these animals at 250 days showed as low a manganese content as those of animals that belonged to the third and 4th generation of manganese deficiency. Ray and Deysach ('42) have observed that mice, when put on a new level of manganese intake, become "saturated" in 40 days. None of the 23 animals of the group to which rats 10, 11, and 12 belonged had showed symptoms of ataxia or incoordination at any time. This suggests that the manganese deficiency must occur during the early development of the animal, in order to produce defects causing ataxia. The mothers of rats 10, 11, and 12 were of the second generation on a 0.1 mg manganese intake. The manganese necessary to prevent deficiency could have been taken in through the milk of the mothers. It seems more likely that with the mothers on this higher level of manganese intake, the manganese required was supplied to the fetuses, and that this was sufficient to carry the young rats far enough through the developmental period to prevent the appearance of ataxia. That differences in fetal manganese content do occur with differences in the

maternal manganese intake is shown by the data of Skinner et al. ('31), although the "low manganese" diet described by these authors contained as much as 0.1 mg manganese per maternal rat per day.

The failure of the ataxic animals to show lesions of the vestibular apparatus which were histologically demonstrable in hematoxylin and eosin- or Weil-stained sections makes it possible to postulate a chemical or enzymatic lesion. Manganese is an activator of numerous enzyme systems — phosphatases, cholinesterase, isocitric dehydrogenase, carboxylase, adenosine triphosphatase (Kleiner, '48) and peptidases (Smith, '49). As soon as sufficient material becomes available, investigation of this chemical hypothesis will be made.

It should be emphasized that the "manganese-deficient" diet of our experiment was not manganese-free. However, it would be difficult to keep rats through more than one generation on a diet much lower in manganese, and a diet that at the same time would be adequate in other respects. Even at the low level of intake of our deficient diet, manganese is very inefficiently retained. The organism is suffering from a lack of manganese, yet, on the 180th day of life, the whole carcass, minus the brain and gastrointestinal tract, contains at most only as much manganese as was ingested during one or two days (table 1). It is not surprising that at the higher levels of manganese intake, presumably adequate, an even smaller proportion of the ingested manganese is retained. There was a rough proportionality between the amount of manganese ingested and that found in the carcass of the 180-day-old rat, although the amount found in the carcasses of the group on a 1 mg daily intake was only about 0.1 and that in the carcasses of the group on a 10 mg daily intake about 0.05 of one day's ration. The data of Skinner et al. ('31) differ somewhat from ours with respect to the fraction of manganese retained. Perhaps this may be explained on the basis of differences in basic ration.

SUMMARY

1. Rats have been raised through 4 generations on a diet otherwise adequate but supplying only 0.03 mg of manganese per day to an adult rat.

2. After the first generation several animals of each generation developed symptoms of ataxia and disturbance in equilibrium which appeared earlier in each successive generation. Slight stimuli greatly exaggerated these symptoms.

3. Serial sections of the temporal bones and brains of these animals, stained with hematoxylin and eosin and according to the method of Weil ('28), failed to reveal any lesions that might be responsible for the symptoms. The presence of a chemical lesion in these areas due to the manganese deficiency is suggested.

ACKNOWLEDGMENTS

The authors wish to acknowledge the valuable technical assistance of Mrs. Evelyn Campbell and Mrs. Lydia Toll.

LITERATURE CITED

BROEK, A., AND L. K. WOLFF 1935 Cu, Zn, and Mn in cow milk. Acta brevia Neerl., *5:* 80. (Through Chem. Abstr., *31:* 3162, 1937.)

BUCHANAN, A. R. 1937 Eye deviation and nystagmus in guinea pigs with lesions of the cerebellum and brain stem. Laryngoscope, *47:* 874.

CASKEY, C. D., AND L. C. NORRIS 1940 Micromelia in adult fowl caused by manganese deficiency during embryonic development. Proc. Soc. Exp. Biol. Med., *44:* 332.

HILL, R. M., D. E. HOLTKAMP, A. R. BUCHANAN AND E. K. RUTLEDGE 1949 Ataxia and loss of equilibrium in manganese-deficient rats. Fed. Proc., *8:* 206.

KING, H. D. 1939 Labyrinthitis in the rat and a method for its control. Anat. Rec., *74:* 215.

KLEINER, I. S. 1948 Human Biochemistry. The C. V. Mosby Company. St. Louis, p. 423.

KUN, E. 1947 Microdetermination of manganese in biological material by means of catalysis. J. Biol. Chem., *170:* 509.

RAY, T. W. 1940 The determination of manganese in organic material containing large amounts of calcium and chlorides. Ibid., *134:* 677.

RAY, T. W., AND L. J. DEYSACH 1942 Storage of manganese by thyroid. Effect on oxygen consumption of the guinea pig. Proc. Soc. Exp. Biol. Med., *51:* 228.

SHERMAN, H. C. 1946 Chemistry of Food and Nutrition. The MacMillan Company, New York, p. 631.

SHILS, M. E., AND E. V. MCCOLLUM 1943 Further studies on the symptoms of manganese deficiency in the rat and mouse. J. Nutrition, *26:* 1.

SKINNER, J. T., W. H. PETERSON AND H. STEENBOCK 1931 The manganese metabolism of the rat. J. Biol. Chem., *90:* 65.

SMITH, E. L. 1949 Catalytic action of the metal peptidases. Fed. Proc., *8:* 581.

WEIL, A. 1928 A rapid method for staining myelin sheaths. Arch. Neur. Psychiat., *20:* 392.

SELF SELECTION OF DIET

XII. EFFECTS OF B VITAMIN DEFICIENCIES ON SELECTION OF FOOD COMPONENTS [1]

E. M. SCOTT, ETHEL L. VERNEY AND PATRICIA D. MORISSEY
Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania

(Received for publication November 21, 1949)

There has been considerable variation reported in the food selections of normal rats when offered the components of a diet (Richter, Holt and Barelare, '38; Scott and Verney, '48), depending upon what choices were offered. Attempts have been made to determine the effects of metabolic disturbances on food selections. Richter ('41) reported a change in the appetite for glucose of adrenalectomized rats but this could not be confirmed (Mark, '42; Clark and Clausen, '43). Warkentin, Warkentin and Ivy ('43) found no effect of experimental thyroid abnormalities on the food selections of rats other than the expected effect on total calories, and Cahill, Dunning and Smith ('43) found no effects of tumors on choice. Richter and Hawkes ('41) attempted to determine the relation of B vitamins to food selection.

The present study is of the effects of B vitamin deficiency on the choice by rats of casein, sucrose, fat and salts, and an investigation of the relation of these choices to nutritional needs.

EXPERIMENTAL

In the first experiment 10 groups of male and 10 groups of female albino rats were used. To minimize genetic differences, each group consisted of 5 litter mates of the same sex

[1] Aided by grants from the Nutrition Foundation, Inc., and from the Buhl Foundation.

and of approximately equal weight when weaned, at 21 to 23 days of age. They were fed a standard diet for a three-week period.[2] Vitamins were given separately as pills [3] in such a manner that one animal in each group served as a control, one was deficient in thiamine, another deficient in riboflavin, a 4th deficient in pyridoxine, and the last deficient in pantothenate.

At the end of three weeks, all animals were given their choice of purified casein, sucrose, hydrogenated vegetable oil, and salt mixture, each in a separate cup. The weights of the

TABLE 1

Growth and food consumption during the control period [1]

TYPE OF ANIMALS	FIRST EXPERIMENT		SECOND EXPERIMENT	
	Body weight change	Food consumed	Body weight change	Food consumed
	gm	*gm*	*gm*	*gm*
Thiamine-deficient	20.5 ± 1.6	76.6 ± 2.3	12.9 ± 2.6	91.8 ± 3.8
Riboflavin-deficient	15.6 ± 0.7	67.6 ± 2.0	9.5 ± 1.3	73.5 ± 2.7
Pyridoxine-deficient	28.4 ± 1.0	83.4 ± 2.3	26.9 ± 1.7	97.5 ± 3.9
Pantothenate-deficient	19.7 ± 0.6	75.0 ± 2.8	16.2 ± 1.1	92.2 ± 2.7
Controls	67.0 ± 2.4	141.7 ± 4.4		

[1] All data given in terms of mean and standard error of the mean.

animals and the amount of each food choice eaten were determined daily, and the cups then interchanged in a predetermined random manner.

In the second experiment, 20 groups of male and 20 groups of female rats were used. Each group consisted of three litter mates of the same sex and of almost equal weight at

[2] The standard diet consisted of 24% purified casein ("Labco vitamin-free"), 10% hydrogenated vegetable oil ("Primex"), 4% salt mixture (Jones and Foster, '42), and 62% sucrose.

[3] One pill was given each rat daily. The pill given the control animal contained 60 μg thiamine hydrochloride, 120 μg thiamine riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola, 1 mg α-tocopherol and 10 mg choline chloride, in a dextrin-powdered sugar base. Thiamine was omitted from this formula to produce deficiency of that vitamin and riboflavin, pyridoxine or pantothenate omitted to produce the other deficiencies.

weaning. They were given for three weeks the same standard diet as in the first experiment, but the administration of the vitamin pills was modified so that 10 groups (5 of each sex) were deficient in thiamine, 10 were deficient in riboflavin, 10 were deficient in pyridoxine and 10 were deficient in pantothenate.

At the end of three weeks, one of the animals in each group was given the standard diet for three more weeks, another a high protein diet and a third a high fat diet,[4] with no other choices. The animals were weighed and food intakes determined three times weekly.

RESULTS

The weight gains and food consumption of all animals during the first three-week period are shown in table 1. The weight gains and food selections of the animals in the first experiment are shown in table 2. As in previous experiments (Scott, '46), it was found that the animals could be sharply divided into a group which ate negligible amounts of protein (group A eating less than 0.25 gm per day), and a group choosing considerable protein (group B). The weight changes of these groups differed considerably. The choices of the deficient animals compared to those of their litter-mate controls are shown in table 3. Here no separation into groups that did or did not eat protein was possible, since several litters had members in both groups. These results show that thiamine-deficient rats substituted fat for protein when compared with controls, that riboflavin-deficient animals may have eaten more carbohydrate, that pyridoxine-deficient animals ate less protein, and that pantothenate-deficient animals substituted sucrose for fat to a large extent.

The results of the second experiment are shown in table 4. In all cases except that of thiamine deficiency the loss in body weight on these mixed diets was less than when no protein was

[4] The compositions of these diets were: High protein — 40% casein, 10% fat, 46% sucrose, 4% salts; High fat — 10% casein, 70% fat, 16% sucrose, 4% salts.

TABLE 2

Food choices of B vitamin-deficient rats

TYPE OF ANIMAL [1]		NUMBER OF ANIMALS	BODY WEIGHT CHANGE [2]	TOTAL CALORIES CONSUMED	PER CENT OF TOTAL CALORIES AS:			SALTS INTAKE
					Sucrose	Fat	Casein	
			gm/day	*Cal.*				*gm/100 Cal.*
Thiamine-deficient	A	20	−0.83 ± 0.04 (2)	216.3 ± 15.0	5.1 ± 1.1	93.3 ± 1.2	1.7 ± 0.5	0.4 ± 0.1
Riboflavin-deficient	A	18	−0.49 ± 0.05 (6)	260.5 ± 23.5	24.2 ± 5.0	74.4 ± 5.5	1.2 ± 0.3	0.6 ± 0.1
	B	2	−0.21 ± 0.31 (1)	244.1 ± 84.0	14.9 ± 8.0	24.0 ± 17.0	61.1 ± 26.0	0.7 ± 0.1
Pyridoxine-deficient	A	19	−0.79 ± 0.07 (2)	314.0 ± 15.1	16.3 ± 6.8	83.0 ± 6.8	0.8 ± 0.1	0.4 ± 0.1
	B	1	0.76 (0)	479.8	1.5	69.4	29.0	0.5
Pantothenate-deficient	A	12	−0.73 ± 0.06 (4)	236.5 ± 25.6	45.1 ± 8.8	53.7 ± 8.9	1.2 ± 0.3	0.4 ± 0.1
	B	8	0.05 ± 0.14 (3)	264.9 ± 30.2	57.6 ± 3.7	21.4 ± 2.8	21.1 ± 3.4	0.7 ± 0.1
Control	A	14	−1.18 ± 0.07 (0)	563.4 ± 35.3	9.5 ± 4.1	89.2 ± 4.4	1.4 ± 0.3	0.3 ± 0.1
	B	6	2.02 ± 0.31 (0)	874.5 ± 74.7	18.6 ± 9.1	44.7 ± 8.6	36.7 ± 6.4	0.9 ± 0.1

[1] Group A selected less than 0.25 gm casein per day; group B ate more than this amount.

[2] Figures in parentheses represent number of animals that died during the experimental period.

selected by animals given a choice in the first experiment. A study of the differences between litter mates shows the following significant points: The high fat diet allowed less weight loss and greater longevity than either the high protein or standard diets, with respect to the thiamine-deficient group. The high fat diet gave greater weight loss than the other two in the riboflavin-deficient group. No consistent effects were noted in the pyridoxine-deficient group, although the high protein diet may have given the greatest weight loss. The pantothenate group showed no definite effect, but the high protein diet may have allowed less weight loss.

TABLE 3

Comparison of food choices of deficient animals with those of controls

COMPONENTS	AVERAGE DIFFERENCE [1] BETWEEN CONTROLS AND —			
	Thiamine-deficient	Riboflavin-deficient	Pyridoxine-deficient	Pantothenate-deficient
	%	%	%	%
Carbohydrate	— 7.1 ± 4.3	11.3 ± 6.8	3.5 ± 7.9	38.4 ± 7.0
Fat	17.4 ± 5.9	— 6.6 ± 9.8	6.9 ± 10.1	— 35.6 ± 9.6
Protein	— 10.2 ± 4.2	— 4.7 ± 6.7	— 10.1 ± 4.4	— 2.8 ± 4.0

[1] Per cent of total calories selected by deficient animals minus per cent of total calories selected by controls.

DISCUSSION

The experiments of Richter and Hawkes ('41) were somewhat difficult to interpret because most of their groups suffered from multiple deficiencies of B vitamins. These authors gave their animals as choices purified casein, sucrose and olive oil (and yeast in the normal group). In our experiments with fats we have found hydrogenated oil to be a very well-liked choice, with other fats much less so (Scott and Verney, '48). Apparently olive oil is not particularly well-liked by rats, and this leads to higher sucrose and casein intakes than we have observed. A conclusion reached by Richter was that B vitamin deficiencies, and in particular thiamine deficiency, led to low protein and high fat intakes. This conclusion is

TABLE 4

Effect of diet composition on weight changes of deficient animals

ANIMALS	DIET	AVERAGE WEIGHT CHANGE	AVERAGE SURVIVAL [1]	AVERAGE FOOD INTAKE	DIFFERENCES [2] IN WEIGHT CHANGE COMPARED WITH:	
					Standard	High protein
		gm/day	*days*	*Cal./day*	*gm/day*	*gm/day*
Thiamine-deficient	Standard	−1.00 ± 0.27	7.4 ± 1.8 (10)	9.7 ± 1.7		
	High protein	−0.86 ± 0.26	7.9 ± 1.8 (10)	7.8 ± 1.1	0.14 ± 0.22 (0.5 ± 2.2)	
	High fat	−0.41 ± 0.16	11.4 ± 2.0 (9)	11.5 ± 1.6	0.59 ± 0.22 (4.0 ± 2.1)	0.45 ± 0.17 (3.5 ± 2.6)
Riboflavin-deficient	Standard	0.11 ± 0.05	(1)	13.6 ± 0.9		
	High protein	0.30 ± 0.13	(3)	14.8 ± 1.0	0.19 ± 0.14	
	High fat	−0.11 ± 0.11	(1)	18.0 ± 1.9	−0.22 ± 0.09	−0.41 ± 0.19
Pyridoxine-deficient	Standard	0.35 ± 0.26	(1)	21.5 ± 2.0		
	High protein	−0.09 ± 0.24	(2)	18.3 ± 2.1	−0.44 ± 0.32	
	High fat	0.03 ± 0.14	(0)	23.4 ± 2.0	−0.32 ± 0.24	0.12 ± 0.25
Pantothenate-deficient	Standard	−0.20 ± 0.08	(3)	16.8 ± 1.2		
	High protein	−0.03 ± 0.15	(5)	18.2 ± 2.1	0.17 ± 0.20	
	High fat	−0.25 ± 0.07	(6)	15.3 ± 0.4	−0.05 ± 0.12	−0.22 ± 0.13

[1] Figures in parentheses represent number of animals out of 10 that died during the experimental period.
[2] Average differences between litter mates. Figures in parentheses are differences in survival times in days.

verified in the present experiment. However, only a single change occurs, since in general when rats do not select protein they select fat instead. This was shown previously (Scott, '46) and is illustrated again in table 2.

There appear to be three possible explanations for the results obtained in this selection experiment. First, it can be postulated that a general characteristic of several B deficiencies is lack of appetite for casein, just as weight loss and early mortality are symptoms common to all such deficiencies. This appears somewhat reasonable if one assumes that casein is distasteful but that some, not all, normal animals learn that it makes them feel better to eat this protein. Animals deficient in B vitamins receive less benefit from protein and consequently might have less motivation to eat it. The casein used here has been reported (Elvehjem et al., '45) to contain no thiamine, 1.2 p.p.m. riboflavin, 0.08 p.p.m. pyridoxine and 0.37 p.p.m. pantothenate. Considering that learned appetites for foods containing as little as 0.1 p.p.m. thiamine have been observed in thiamine-deficient animals (Scott and Verney, '49), it is entirely possible that the appetites for casein shown by riboflavin- and pantothenate-deficient animals may be appetites for the vitamins this protein contains.

The second possible explanation is that the changes in appetite are more directly related to needs in terms of components of the diet. It was indicated in previous experiments that such relationships in normal animals are dubious since the appetites studied varied considerably, depending on the particular fat, protein, or carbohydrate under consideration (Scott and Verney, '48). Although there is no reason *a priori* why the appetites of deficient animals should be more closely related to needs than those of normal animals, the second experiment was designed to test this hypothesis. The high fat diet used therein approximated the average composition of the diets selected by all animals in the first experiment except the pantothenate-deficient group. It was found that thiamine-deficient animals benefited from the high fat diet, and in fact selected somewhat more fat than the other groups;

and that riboflavin-deficient animals were harmed by the high fat diet and had a slightly low intake of fat.

Although the effect on weight change of a high protein intake when compared with a low protein intake was relatively small, as is shown in table 4, the data in table 2 and a comparison of tables 2 and 4 indicate a marked difference in weight change, in all cases except thiamine deficiency, between those animals which ate protein and those that ate virtually none. Still, most of the animals with a choice selected essentially no protein. Furthermore, the most marked change in appetite, that of pantothenate-deficient animals with respect to sucrose and fat, had no apparent benefit. Consequently, not all the changes in appetite of these animals could be related directly to needs. It is possible that these changes in appetite may have been learned, and that they resulted from the animals' deriving a feeling of well-being on eating certain food choices. For this explanation to be correct, it must be assumed that the rat's subjective state bears no necessary relation to his ultimate welfare, at least insofar as the latter can be judged from such indexes as weight change and survival.

A third possible explanation appears to be simpler than these. Our results may be simply flavor effects. From human experience it can be concluded that illness can cause changes in the subjective response to food, and it is at least believed that such changes in response are selective, applying more to one food than another. It is only necessary to assume that the malaise of these deficient animals results in certain foods' being disliked more than usual, and others are then eaten instead.

SUMMARY

When rats deficient in thiamine, riboflavin, pyridoxine or pantothenate were allowed their choice of casein, sucrose, hydrogenated fat, or salt mixture, certain specific differences between their food selections and those of control animals were observed. The thiamine-deficient group selected more fat and less protein that the controls; the pyridoxine-deficient

group selected less protein; and the pantothenate group much less fat and more carbohydrate than the controls. Possible explanations of these results are considered.

LITERATURE CITED

CAHILL, W. M., W. F. DUNNING AND A. H. SMITH 1943 A free choice dietary study of tumor bearing rats. Cancer Res., *3:* 830.

CLARK, W. G., AND D. F. CLAUSEN 1943 Dietary "self-selection" and appetite of untreated and treated adrenalectomized rats. Am. J. Physiol., *139:* 70.

ELVEHJEM, C. A., M. D. CANNON AND R. K. BOUTWELL 1945 Vitamin content of casein. Science, *102:* 529.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

MARK, J. 1942 Inactivation of desoxycorticosterone acetate. Endocrinology, *31:* 582.

RICHTER, C. P. 1941 Sodium chloride and dextrose appetite of untreated and treated adrenalectomized rats. Ibid., *29:* 115.

RICHTER, C. P., AND C. D. HAWKES 1941 The dependence of carbohydrate, fat and protein appetite of rats on the various components of the vitamin B complex. Am. J. Physiol., *131:* 639.

RICHTER, C. P., L. E. HOLT AND B. BARELARE, JR. 1938 Nutritional requirements of normal growth and reproduction in rats studied by the self selection method. Ibid., *122:* 734.

SCOTT, E. M. 1946 Self selection of diet. I. Selection of purified components. J. Nutrition, *31:* 397.

SCOTT, E. M., AND E. L. VERNEY 1948 Self selection of diet. VIII. Appetite for fats. Ibid., *36:* 91.

———— 1949 Self selection of diet. IX. The appetite for thiamine. Ibid., *37:* 81.

WARKENTIN, J., C. WARKENTIN AND A. C. IVY 1943 The effect of experimental thyroid abnormalities on appetite. Am. J. Physiol., *139:* 139.

THE ARGININE AND HISTIDINE CONTENT OF MEATS[1]

R. J. SIRNY, I. T. GREENHUT AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication February 6, 1950)

Microbiological methods are now widely used for the determination of amino acids in natural materials. These methods were first applied to meats in our laboratory when Schweigert, McIntire, Elvehjem and Strong ('44) determined leucine and valine in these materials. Subsequent work on amino acids in meats resulted in reports on leucine, isoleucine and valine (Schweigert et al., '45), tryptophan (Greenhut et al., '46a, '47), threonine (Greenhut et al., '46b), phenylalanine (Greenhut et al., '47) and lysine, methionine and threonine (Greenhut et al., '48). The present report on arginine and histidine completes the analyses of meats with respect to their content of the 10 amino acids which have been designated as the essential amino acids.

The assay procedures employed for arginine and histidine were similar to those used in previous work. Additional evidence for the reliability of these methods was obtained by the use of two or more organisms in duplicate or triplicate assays conducted simultaneously. *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R and *Lactobacillus delbrueckii* 3 were used in analyses for both arginine and histidine.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Livestock and Meat Board.

EXPERIMENTAL

Preparation of samples

Two separate lots of meat samples, representing fresh cuts of beef, lamb, pork, and veal and whole beef organs (tables 2 and 3) were used. The samples in lot I were the same as those used in the previously reported work on lysine,

TABLE 1

Release of arginine and histidine from beef round under varying conditions of hydrolysis

(Values in grams per 100 gm crude protein, calc. to 16% N)

CONCENTRATION OF HCl, LENGTH OF HYDROLYSIS AND VOLUME OF ACID [1]	ARGININE	HISTIDINE	
	L. delbrueckii 3	*L. delbrueckii*	*L. mesenteroides*
2N, 5 hr., 100 ml	6.3	3.7	3.4
2N, 5 hr., 40 ml	6.7	3.6	3.3
2N, 10 hr., 40 ml	6.9	3.4	3.4
2N, 15 hr., 40 ml	6.8	3.3	3.3
2N, 20 hr., 40 ml	6.5	3.2	3.2
2N, 25 hr., 40 ml	6.3	3.1	3.3
3N, 5 hr., 40 ml	6.7	3.4	3.4
3N, 10 hr., 40 ml	6.0	3.2	3.2
3N, 15 hr., 40 ml	..	3.4	3.5
3N, 20 hr., 40 ml	6.5	3.4	3.5
3N, 25 hr., 40 ml	6.3	3.2	3.3
4N, 5 hr., 40 ml	6.3	3.4	3.5
4N, 10 hr., 40 ml	6.1	3.3	3.5
4N, 15 hr., 40 ml	5.9	3.2	3.5
6N, 24 hr., reflux	5.6	3.5	3.6

[1] For 5 gm fresh meat, or approximately 1 gm crude protein.

methionine and threonine. All analyses were made simultaneously on the same hydrolysates and the treatment and preparation of the samples have been described by Greenhut et al. ('48).

The samples in lot II were obtained at a later date but were treated in a similar manner. They were selected on the killing floor of a local packing house to insure freshness, and special precautions were taken to insure that the various cuts within a species and the duplicates of the different beef

organs came from different animals. The entire portions of each meat cut and organ were trimmed of excess fat, bone and gristle, and were finely ground in an electric meat grinder. The preparations were made as homogeneous as possible by passing them through the grinder several times. Since uncontrolled moisture losses may cause considerable variation in the amounts of total solids present in fresh, wet materials, the samples for nitrogen analyses and for hydrolysis were weighed out simultaneously. Nitrogen determinations were done by a macro-Kjeldahl procedure. Hydrolysates of the meats were prepared by autoclaving 5-gm samples (containing approximately 1 gm of crude protein) with 40 ml of 2N HCl for 5 hours at 121°C. in covered Erlenmeyer flasks.

In preliminary work (table 1) a study of the liberation of these amino acids from a sample of beef round had been conducted, with treatment of the beef round being the same as that described above except that the conditions of hydrolysis were varied as shown in the table.

All hydrolysates were filtered at room temperature, diluted to a convenient volume, i.e., 200 ml, and stored at 5°C. until amino acid analyses could be made. Prior to analyses, appropriate aliquots of the hydrolysate were neutralized with the aid of a pH meter and further diluted to the concentration required for the desired microbiological response in the assay.

Samples of the leg of lamb in lot II were also prepared for hydrolysis by drying from the frozen state and removal of fat by ether extraction. Lyman et al. ('46) have suggested the use of dried, fat-free meat samples for microbiological analyses, and it was desired to compare results with samples so prepared with the results obtained under our conditions. The drying procedure was facilitated by the use of lyophilization equipment [2] which permitted the rapid drying in 8 to 12 hours of 50-gm meat samples. The procedure consisted essentially of preparing a water slurry of the meat sample

[2] Kindly made available by Dr. H. H. Sommer and associates, Department of Dairy Industry, University of Wisconsin, who have designed and built this equipment in a single unit capable of handling 4 preparations simultaneously.

with a Waring Blendor, freezing it in a thin layer on the inner walls of a 2-l round bottom flask by rotation of the flask in a dry ice-alcohol freezing mixture, then attaching the flask to the high vacuum source ($< 50\,\mu$) and removing the water vapors by condensation and freezing in a trap with the freezing mixture mentioned above. When drying is complete, the dry shell of meat in the flask is broken up and ground with a mortar and pestle. This yields a uniform, finely divided preparation that possesses excellent keeping qualities at room temperature and which is well suited for ether extraction by Soxhlet procedures.

Assay procedures

The microbiological techniques and assay medium employed in analyses for arginine and histidine in the samples of lot I were precisely the same as those previously reported by Greenhut et al. ('48). The amino acid being determined was omitted from the medium, and *L. delbrueckii* 3, *S. faecalis* and *L. mesenteroides* were used as the assay organisms.

For analyses of the samples in lot II, the uniform medium of Henderson and Snell ('48) was adopted, with only slight modification in certain of the procedures described by these workers. The sterilization time was 10 minutes at 121°C. in the autoclave to avoid excessive caramelization of the media. Standard solutions were prepared to contain the equivalent of 20 µg L-arginine and 10 µg L-histidine per milliliter, the former being added as the monohydrochloride and the latter as the monohydrochloride monohydrate, for assays with *L. delbrueckii* 3 and exactly one-half of these concentrations for assays with *S. faecalis* and *L. mesenteroides*. Standard curves were established by the use of 5 different levels of amino acid in each 6-tube row. A minimum of two rows of standard tubes per 10-row rack was used in all assays. Samples were added at 6 different levels per row, such that the highest concentration represented a 5-fold increase in concentration over the lowest level. It is felt that this modification has

the advantage of revealing drift, i.e., progressively increasing or decreasing values with increasing sample concentration, which might pass undetected if fewer levels were used. Since drift is indicative of stimulation or inhibition by constituents of the sample which are not present in the standard, or are present in greater amounts, it is evident that drifting values will result in erroneous results. This procedure has the further advantage that when the amino acid content of the materials under analysis is not known, or can be only roughly estimated, the 5-fold range in sample concentration provides a much greater chance of striking the standard curve with the estimated concentration.

RESULTS AND DISCUSSION

It will be seen from tables 2 and 3 that *L. delbrueckii* 3 served as the primary assay organism for both arginine and histidine. Since at present the taxanomic identity of *L. delbrueckii* is rather controversial, it becomes essential to present a brief history of our organism for the purpose of identifying it with respect to some of the studies in which this particular strain has been utilized.

The original culture at the University of Wisconsin was obtained in 1926 by Dr. E. B. Fred of the Department of Bacteriology from Dr. E. Pribram of Kral's bakteriologische Museum in Vienna, and was presumably an original isolation by Henneberg, after whose name this strain was designated as *Lactobacillus delbrueckii III Henneberg.* It was used by Tatum and Peterson ('35) in their development of a fermentation method for production of dextro-lactic acid. Guirard et al. ('46) reported that this organism gave values for histidine in excellent agreement with those obtained with *L. mesenteroides.* In our laboratory it has been used extensively for the microbiological estimation of phenylalanine, isoleucine, leucine and valine, as well as of arginine and histidine. Under our conditions, it also possesses a definite requirement for glutamic acid, tryptophan and tyrosine, although good possibilities for use in assay work are apparent only in the

TABLE 2

Analyses of meats in lot I by various organisms

MEAT	PROTEIN	ARGININE								HISTIDINE							
		LD-3		L. mes.		S. faec.		Mean by all 3 organisms	Amt. in meat	Retention	LD-3		L. mes.		S. faec.		Mean by all 3 organisms	Amt. in meat	Retention
	%	no.[1]	%[2]	no.	%	no.	%	%	%[2]	%	no.	%	no.	%	no.	%	%	%	%
Veal																			
Shank	21.7	4	6.9			1	7.4	7.0	1.52		5	2.8			1	2.9	2.8	0.61	
Shoulder	20.4	5	6.7			1	6.2	6.6	1.35		8	3.3			2	3.4	3.3	0.67	
Roast shoulder	27.4	5	6.6			1	6.5	6.6	1.81	102	6	2.9	1	3.0	4	3.0	2.9	0.79	90
Loin	17.1	3	6.7	1	7.0	2	7.0	6.8	1.16		7	3.6	1	3.7	4	3.6	3.6	0.62	
Round	20.4	6	6.5	2	7.3	2	7.1	6.8	1.39		6	3.3	2	3.2	3	3.7	3.4	0.69	
Roast round	29.0	3	6.4			2	6.6	6.5	1.89	100	7	3.2	2	2.9	3	3.1	3.1	0.90	96
Group mean								6.7									3.2		
Lamb																			
Shank	11.7	7	6.5	2	7.3	4	7.4	6.9	0.81		9	3.1	3	3.1	2	3.1	3.1	0.36	
Roast shank	12.5	7	6.7	3	6.6	3	7.2	6.8	0.85	98	7	2.7	1	2.9	3	2.8	2.7	0.34	89
Shoulder	15.8	3	6.1	1	6.1	1	6.5	6.2	0.98		6	3.0			1	3.0	3.0	0.47	
Loin	13.0	3	6.2			1	6.5	6.3	0.82		3	2.9	1	3.3	1	3.3	3.1	0.40	
Leg	16.5	2	6.5	1	6.6	3	6.8	6.7	1.11		6	2.6	2	2.7	4	2.9	2.7	0.46	
Roast leg	27.5	3	6.6			1	6.4	6.6	1.82	95	4	2.4			2	2.7	2.5	0.69	87
Group mean								6.6									2.9		
Pork																			
Picnic ham	16.5	3	6.7	1	6.4	2	6.6	6.6	1.09		6	3.4	1	3.0	3	3.6	3.4	0.56	
Butt	12.2	2	6.6					6.6	0.81		5	3.1	2	3.1	1	3.1	3.1	0.38	
Roast butt	22.8	5	6.9	1	7.6	3	7.4	7.1	1.62	107	7	3.0	1	3.3	3	3.0	3.0	0.68	96
Loin	17.4	3	6.7	1	6.5	2	6.5	6.6	1.15		6	3.5	1	3.9	3	3.8	3.6	0.63	
Ham	13.9	4	6.8	1	6.0	3	6.6	6.6	0.92		5	3.8	2	3.7	6	4.0	3.9	0.54	
Roast ham	27.6	1	6.8	1	6.5	2	6.6	6.6	1.82	98	5	3.2			2	3.3	3.2	0.88	79
Group mean								6.7									3.4		
Beef																			
								6.8	1.18		4	3.7			1	4.1	3.8	0.66	

Ham	13.9	4	6.8	1	6.0	3	6.6	6.6	0.92		5	3.8	2	3.7	6	4.0	3.9	0.54	
Roast ham	27.6	1	6.8	1	6.5	2	6.6	6.6	1.82	98	5	3.2			2	3.3	3.2	0.88	79
Group mean								6.7									3.4		
Beef																			
Chuck	17.4	3	6.8					6.8	1.18		4	3.7			1	4.1	3.8	0.66	
Roast chuck	22.7	4	6.9	1	6.2	2	7.0	6.8	1.54	103	5	3.0			3	3.1	3.0	0.68	81
Rib	16.7	2	6.3			1	6.2	6.3	1.05		5	3.6			1	3.5	3.6	0.60	
Loin end	17.6	1	6.7			2	6.1	6.5	1.14		3	3.3			1	3.5	3.3	0.58	
Round	20.6	3	7.0	1	5.8	2	6.6	6.7	1.38		5	3.3	1	3.2	2	3.5	3.3	0.68	
Roast round	33.4	1	5.6	1	6.2	2	6.2	6.1	2.03	94	6	3.2	2	3.3	3	3.0	3.2	1.07	101
Group mean								6.5									3.4		
Brain	10.6	4	6.1			1	5.8	6.0	0.64		8	2.7	3	2.8	5	2.7	2.7	0.29	
Brain	10.8	4	6.0			1	6.6	6.1	0.66		6	2.3	1	2.6	4	2.8	2.5	0.27	
Brain	10.3	1	6.5			1	6.5	6.5	0.67		4	2.3	1	2.5	3	2.6	2.4	0.25	
Mean								6.2									2.5		
Heart	17.4	3	6.9					6.9	1.20		5	2.7			2	2.6	2.7	0.47	
Heart	17.7	3	6.8					6.8	1.20		4	2.8			1	2.8	2.8	0.50	
Mean								6.8									2.8		
Kidney	16.7	4	6.5	2	6.2	3	6.2	6.3	1.05		6	2.6	1	2.6	2	2.6	2.6	0.43	
Kidney	16.4	3	6.5			1	6.6	6.5	1.07		4	2.8			1	3.0	2.8	0.46	
Kidney	16.0	4	6.0	1	5.9	1	6.0	6.0	0.96		4	2.4	1	2.5	4	2.7	2.5	0.40	
Mean								6.3									2.6		
Liver	21.0	3	6.1	1	6.4	1	6.1	6.2	1.30		6	2.8					2.8	0.59	
Liver	20.8	4	6.3	2	6.2	2	6.7	6.4	1.33		3	2.8					2.8	0.58	
Liver	20.4	3	6.1	1	5.6	2	6.7	6.2	1.26		3	2.8	1	2.9	2	2.8	2.8	0.57	
Mean								6.3									2.8		
Tongue	20.5	3	6.4			1	6.0	6.3	1.29		5	2.4	1	2.1	3	2.5	2.4	0.49	
Tongue	20.2	3	6.2			2	6.4	6.3	1.27		6	2.4					2.4	0.48	
Mean								6.3									2.4		
Mean of all analyses			6.4		6.5		6.8					3.0		3.0		3.1			
Standard deviation of all analyses			0.35		0.58		0.57					0.42		0.40		0.54			
Coefficient of variation			5.4		8.9		8.5					14.1		13.2		17.3			

[1] Number as used throughout refers to number of individual analyses represented in the presented values.
[2] Per cent as used throughout signifies grams amino acid per 100 gm meat, or per 100 gm meat protein calc. to 16% N.

TABLE 3

Analyses of meats in lot II

MEAT	CRUDE PROTEIN	ARGININE			HISTIDINE					
		LD-3			LD-3		L. mes.		Mean from 2 organisms	In meat
			In protein	In meat		In protein		In protein		
	%	no.	% [1]	% [1]	no.	%	no.	%	%	%
Veal round	21.3	5	6.1	1.30	3	3.3	3	3.3	3.3	0.70
stew meat	20.6	5	6.6	1.36	3	2.7	3	2.9	2.8	0.58
Group mean			6.4						3.0	
Lamb stew meat	18.6	5	6.5	1.21	3	2.5	3	2.6	2.6	0.48
leg	18.2	5	6.4	1.16	3	2.6	3	2.4	2.5	0.46
leg, dried	57.8	2	6.4	1.16	2	2.5	2	2.4	2.5	0.46
leg, dried and extr.	91.6	2	6.3	1.15	2	2.5	2	2.3	2.4	0.44
Group mean			6.4						2.5	
Pork picnic ham	17.4	5	6.4	1.11	3	2.9	3	2.8	2.9	0.50
loin	20.3	5	6.2	1.26	3	4.0	3	3.6	3.8	0.77
Group mean			6.3						3.3	
Beef round	19.4	5	6.2	1.20	3	3.6	3	3.4	3.5	0.68
chuck	19.6	5	6.1	1.19	3	3.6	3	3.4	3.5	0.69
Group mean			6.2						3.5	
Beef brain	11.1	5	5.9	0.66	3	2.3	3	2.6	2.5	0.28
brain	11.0	4	5.9	0.65	2	2.4	2	2.5	2.5	0.28
Mean			5.9						2.5	
heart	18.1	5	6.5	1.17	3	2.5	3	2.5	2.5	0.46
heart	17.4	4	6.5	1.13	2	2.6	2	2.5	2.6	0.45
Mean			6.5						2.5	
kidney	16.7	5	6.0	1.00	4	2.4	3	2.3	2.4	0.40
kidney	16.6	4	6.1	1.01	3	2.3	2	2.2	2.3	0.38
Mean			6.0						2.3	
liver	18.7	5	6.2	1.16	4	2.6	3	2.6	2.6	0.48
liver	19.7	4	6.2	1.22	3	2.7	2	2.5	2.6	0.51
Mean			6.2						2.6	
tongue	15.3	5	6.6	1.01	4	2.5	2	2.4	2.5	0.38
tongue	14.6	4	6.7	0.98	3	2.5	1	2.4	2.5	0.37
Mean			6.6						2.5	
Mean of all analyses			6.3			2.8		2.8		
Standard deviation of all analyses			0.41			0.52		0.46		
Coefficient of variation			6.5			18.7		16.7		

[1] Per cent as used throughout signifies grams amino acid per 100 gm meat, or per 100 gm meat protein calc. to 16% N.

case of the latter organism.[3] Cystine and serine are stimulatory for this organism, though not specifically required. In its amino acid needs it closely resembles *Lactobacillus casei* 7469, but is notably different in its absolute requirement for histidine.

The results of preliminary studies on meat hydrolysis by HCl are shown in table 1. It can be seen that the microbiological response to arginine is slightly depressed as more drastic conditions of hydrolysis, i.e., higher acid concentration or longer periods of time, are used. While the data are too limited for us to conclude that some destruction of arginine has taken place with the 4N HCl, 15-hour hydrolysis or with the 6N HCl, 24-hour reflux, for which the values are 5.9 and 5.6%, respectively, a definite downward trend is seen with increasing severity of hydrolysis. Under the same conditions the results for histidine are seen to be very constant with both *L. delbrueckii* 3 and *L. mesenteroides*. In this connection it is desirable to mention that the results of other work[4] in progress in this laboratory suggest that for certain other amino acids — e.g., phenylalanine — hydrolysis with 2N HCl for 5 hours is inadequate when certain organisms are used for the assays. Differences in results obtained with different organisms tend to be eliminated or reduced when more intense hydrolysis is used. However, as will be seen in tables 2 and 3, the results given by the three organisms for both arginine and histidine are in close agreement. This would indicate further that the conditions (2N HCl, 5 hours) used for hydrolysis of the meats in this work are adequate and satisfactory for the determination of arginine and histidine in the hydrolysates. Recoveries of the amino acids added prior to hydrolysis under these conditions were also satisfactory.

Results with different organisms

The data in tables 2 and 3 are presented to permit a comparison of the results obtained with the three different or-

[3] Data to be published later by R. G. Chitre and others.

[4] To be published later by M. Klungsøyr and others.

ganisms in repeated analyses of the individual samples. The means of all analyses with each organism have been presented only to aid in this comparison of the three organisms, even though they may not be strictly comparable because not all samples and fewer analyses are represented in the data obtained with *L. mesenteroides* and *S. faecalis*. Standard deviations and coefficients of variability have been calculated on all analyses and therefore include a measure of the over-all variation among repeated analyses of each sample as well as among all samples. In the case of histidine, where actual differences were found between samples, the deviations would necessarily be greater.

For arginine, in the crude protein, the mean values of 6.4, 6.5 and 6.8% with *L. delbrueckii* 3, *L. mesenteroides* and *S. faecalis*, respectively, reflect the generally good agreement between the two former organisms, and the frequently higher values obtained with *S. faecalis*, which may be seen by inspection of the results on the individual samples of lot I. In the case of histidine, excellent agreement among all three organisms is seen both in the individual data and the over-all means of 3.0, 3.0 and 3.1% with *L. delbrueckii* 3, *L. mesenteroides* and *S. faecalis*, respectively, for lot I samples, and the 2.8 and 2.8% values with the two former organisms for the samples of lot II.

Throughout the course of this work it has been noted that a higher degree of variation was encountered in the case of arginine in results with both *L. mesenteroides* and *S. faecalis* as compared to that obtained with *L. delbrueckii* 3. The coefficients of variation of 5.4, 8.9 and 8.5% for *L. delbrueckii* 3, *L. mesenteroides* and *S. faecalis*, respectively, support these observations and have led us to consider *L. delbrueckii* 3 the organism of choice for the determination of arginine under our conditions. In histidine analyses, the coefficients of variation are 14.1, 13.2 and 17.3% for these three organisms in lot I and 18.7 and 16.7% for *L. delbrueckii* 3 and *L. mesenteroides*, respectively, in lot II. The variation appears to be quite similar for *L. delbrueckii* 3 and *L. mesenteroides*,

though higher, as in the case of arginine, with *S. faecalis*. These data on variation support our feeling that *L. delbrueckii* 3 and *L. mesenteroides* give equal satisfaction in the determination of histidine, and are to be preferred as assay organisms under our conditions.

Arginine and histidine in meats

One of the earliest reports on arginine and histidine in muscle was by Hart in 1901, who analyzed a partially purified preparation of ox muscle by chemical precipitation methods and reported a content of 5.1% arginine and 2.7% histidine in the protein. While these results are not directly comparable to those presented in table 4, it is of interest to point out their close agreement with our results of 6.4 and 3.4%, respectively, for arginine and histidine in beef muscle.

In table 4 it may be seen that no large differences were found among the samples of muscle tissues of beef, lamb, pork and veal and among the various beef organs in their content of arginine. Beef brain was among the lowest values at 6.1%, while the muscle tissues gave higher values of 6.4 to 6.5%. A rather wide disagreement exists among the values, in the literature, ranging from 3.4 to 7.9% arginine. However, our results are in good agreement with the more recent work of Schweigert et al. ('49) and Lyman and Kuiken ('49).

The histidine content of the different meats varies considerably among the muscle tissues and is lower in all of the beef organs. Lyman et al. ('47, '49) have made a thorough investigation of similar differences and have attributed them to the occurrence of histidine in the muscles in the non-protein form of carnosine, the beta-alanine peptide of histidine. They further make reference to the work of Fuller et al. ('47) which indicates that the carnosine content of muscle may vary with the histidine content of the ration. In view of this report, the high values for histidine of 3.4, 3.3 and 3.1%, respectively, in beef, pork, and veal may be explained on the basis of carnosine content. Lamb muscle, with a histidine

TABLE 4

Summary of results and comparison with literature values
(All results expressed in grams per 100 gm crude protein, calc. to 16% N)

MEAT	ARGININE			HISTIDINE		
	Our average values	Literature values		Our average values	Literature values	
		Chemical analyses	Microbiological analyses		Chemical analyses	Microbiological analyses
Beef muscle	6.4	7.5,[1] 6.9 [3]	5.9,[3] 3.4,[4] 6.2,[5] 7.9 [7] 6.3,[9] 5.8 [10]	3.4	1.8,[1] 2.25 [3]	3.4,[3] 1.9,[4] 3.7,[5] 2.0 [6] 3.7,[9] 3.8 [10]
Lamb muscle	6.5	7.6 [3]	6.2,[5] 6.7 [8]	2.7	2.4 [3]	2.1,[5] 2.6 [8]
Pork muscle	6.5	6.6 [3]	6.4,[8] 6.0 [8]	3.3	2.2 [3]	3.8,[5] 3.2 [8]
Veal muscle	6.5	7.5 [3]	..	3.1	2.4 [3]	..
Beef brain	6.1	6.4 [3]	6.0 [5]	2.5	2.5 [3]	2.6 [5]
Beef heart	6.7	7.4 [3]	6.4 [5]	2.7	2.1 [3]	2.6 [5]
Beef kidney	6.2	6.9 [3]	6.1 [5]	2.5	2.3 [3]	2.6 [5]
Beef liver	6.3	6.6 [3]	5.8 [9]	2.7	2.0 [3]	2.6 [5]
Beef tongue	6.5	..	..	2.5	..	..

[1] Osborne and Jones ('09).
[3] Beach et al. ('43).
[3] Hier et al. ('45).
[4] Stokes et al. ('45).
[5] Lyman and Kuiken ('49).
[6] Horn et al. ('48a).
[7] Horn et al. ('48b).
[8] Schweigert et al. ('49).
[9] Dunn et al. ('49a).
[10] Neilands et al. ('49).

content of 2.7%, would appear to contain less carnosine and resembles the beef organs in the total amount of histidine that is nutritionally available.

Comparison of the histidine results with the literature values again shows marked disagreement. Part of this disagreement may be due to variations in carnosine content, especially in those cases where water-extracted preparations such as those used by Beach et al. ('43) were employed for the analyses. As in the case of arginine, our results are in good agreement with those of Schweigert et al. ('49) and Lyman and Kuiken ('49), as well as with those of Beach et al. ('43) for lamb muscle and the beef organs.

In the foregoing discussion, all data have been expressed in the conventional percentage of the crude protein or, more correctly, in grams of amino acid per 100 gm of crude protein, based on protein containing 16% nitrogen. In tables 2 and 3, data are also given for the amounts of arginine and histidine in the meat itself. While these values vary with the protein content of the meat, which in turn generally reflects the fat content, they are of interest from the standpoint of determining the actual amino acid intake in a diet or ration.

Also in table 3 are the analyses of the dried, and dried and ether-extracted, samples of leg of lamb. The values are 6.4 and 6.3%, respectively, for arginine and 2.5 and 2.4% for histidine, comparing favorably with the 6.4 and 2.5% arginine and histidine in the undried preparations. While the dried preparations are much easier to work with and possess good keeping qualities, it is felt that little is gained in accuracy of microbiological assay results over the routine procedure of hydrolyzing fresh meats directly for analyses.

The results of this survey give additional evidence for the oft-repeated observation concerning the marked similarity in amino acid composition among the muscle tissues and some of the organs of various species and different stages of maturity of animals. With reference to previous analyses for the amounts of the other 8 amino acids of prime nutritional importance, together with the present results, the pat-

tern of distribution of these 10 amino acids in muscle appears very similar, with only isolated cases of difference among individual samples in the analyses. Although this similarity does not extend as completely to all results with various animal organs, the pattern is not greatly divergent from that in muscle. The most striking deviation is the case of lysine in beef brain, for which the values are about 20% lower than those for muscles. Whether or not these differences indicate actual variations in protein composition, or different proportions of different proteins, is not known. Further evidence of muscle protein similarity is the recent work of Dunn et al. ('49b), who have studied the distribution of 12 amino acids in 8 animal species not investigated in our present work, and who have concluded that "the 12 amino acids were distributed similarly in the protein of the whole animals, organs, and tissues investigated."

Retention of arginine and histidine

The per cent retention of these amino acids is also indicated in table 2, and represents the total amount of amino acid retained by the particular cut of meat after being roasted according to normal roasting procedures in an electric roaster. The retention is then expressed as a per cent of the total amount of amino acid present before roasting, based on analysis of a fresh portion of the same cut of meat, with due allowance made for shrinkage of the meat during roasting. It may be seen that retention of arginine is essentially complete, ranging from 94 to 107%. Retention of histidine is considerably lower, ranging from 79 to 101%. It has not been determined whether or not these lower retentions reflect loss of histidine in the form of carnosine during the roasting process, though this is a very likely possibility. However, the retention of histidine is also low in the case of the leg and shank of lamb, being 87 and 89%, respectively. Since these cuts of lamb are presumed to be lower in carnosine, the possibility that there are other histidine losses is not completely eliminated.

SUMMARY

The arginine and histidine content of 55 different samples of beef, lamb, pork and veal muscle cuts and beef organs have been determined by microbiological analyses with three different organisms, *L. delbrueckii* 3, *L. mesenteroides* P-60 and *S. faecalis* R. Results with all three organisms were in good agreement with each other in repeated analyses, thus serving as an indication of the reliability of the procedures used. However, evaluation of the variation encountered in analyses with these organisms has led to consideration of *L. delbrueckii* 3 as the organism of choice for the assay of arginine and of *L. delbrueckii* 3 and *L. mesenteroides* as preferred organisms for the estimation of histidine. Values for arginine in meats range from 6.1% of the crude protein in beef brain to 6.7% in beef heart. Wider differences are encountered in histidine analyses, ranging from 2.5% in beef brain, kidney and tongue to 3.4% in beef muscle, the higher values presumably reflecting a high carnosine content in the muscles. Retention of arginine during the roasting of meats ranges from 94 to 107%, while that of histidine ranges from 79 to 101%. The lower retention of histidine may be due to loss of carnosine during the cooking process.

LITERATURE CITED

BEACH, E. F., B. MUNKS AND A. ROBINSON 1943 The amino acid composition of animal tissue protein. J. Biol. Chem., *148:* 431.

DUNN, M. S., M. N. CAMIEN, S. EIDUSON AND R. B. MALIN 1949a The nutritive value of canned foods. I. Amino acid content of fish and meat products. J. Nutrition, *39:* 177.

DUNN, M. S., M. N. CAMIEN, R. B. MALIN, E. A. MURPHY AND P. J. REINER 1949b Percentages of 12 amino acids in blood, carcass, heart, kidney, liver, muscle, and skin of 8 animals. Univ. of Cal. Publ. in Physiol., *8:* 293.

FULLER, A. T., A. NEUBERGER AND T. A. WEBSTER 1947 Histidine deficiency in the rat and its effect on the carnosine and anserine content of muscle. Biochem. J., *41:* 11.

GREENHUT, I. T., R. L. POTTER AND C. A. ELVEHJEM 1947 The phenylalanine and tryptophane content of meats. Arch. Biochem., *15:* 459.

GREENHUT, I. T., B. S. SCHWEIGERT AND C. A. ELVEHJEM 1946a Hydrolysis procedures for the determination of tryptophane in proteins and foodstuffs by the microbiological procedure. J. Biol. Chem., *165:* 325.

———— 1946b The amino acid requirements of *Streptococcus faecalis* and the use of this organism for the determination of threonine in natural products. Ibid., *162:* 69.

GREENHUT, I. T., R. J. SIRNY AND C. A. ELVEHJEM 1948 The lysine, methionine and threonine content of meats. J. Nutrition, *35:* 689.

GUIRARD, B. M., E. E. SNELL AND R. J. WILLIAMS 1946 Microbiological determination of amino acids. IV. Lysine, histidine, arginine and valine. Proc. Soc. Exp. Biol. Med., *61:* 158.

HART, E. 1901 Ueber die quantitative Bestimmung der Spaltungsprodukte von Eiweisskorpern. Zschr. phys. Chem., *33:* 347.

HENDERSON, L. M., AND E. E. SNELL 1948 A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem., *172:* 15.

HIER, S. W., C. E. GRAHAM, R. FRIEDES AND D. KLEIN 1945 The microbiological determination of amino acids in animal proteins. Ibid., *161:* 705.

HORN, M. J., D. B. JONES AND A. E. BLUM 1948a Microbiological determination of histidine in proteins and foods. Ibid., *172:* 149.

———— 1948b Microbiological determination of arginine in proteins and foods. Ibid., *176:* 59.

LYMAN, C. M., B. BUTLER, O. MOSELEY, S. WOOD AND F. HALE 1946 The methionine content of meat. Ibid., *166:* 173.

LYMAN, C. M., AND K. A. KUIKEN 1949 The amino acid composition of meat and some other foods. Tex. Agric. Exp. Sta. Bull. 708.

LYMAN, C. M., K. A. KUIKEN AND F. HALE 1947 The histidine content of meat. J. Biol. Chem., *171:* 233.

NEILANDS, J. B., R. J. SIRNY, I. SOHLJELL, F. M. STRONG AND C. A. ELVEHJEM 1949 The nutritive value of canned foods. II. Amino acid content of fish and meat products. J. Nutrition, *39:* 187.

OSBORNE, T. B., AND D. B. JONES 1909 Hydrolysis of ox muscle. Am. J. Physiol., *24:* 437.

SCHWEIGERT, B. S., B. T. GUTHNECK, H. R. KRAYBILL AND D. A. GREENWOOD 1949 The amino acid composition of pork and lamb cuts. J. Biol. Chem., *180:* 1077.

SCHWEIGERT, B. S., J. M. MCINTIRE, C. A. ELVEHJEM AND F. M. STRONG 1944 The direct determination of valine and leucine in fresh animal tissues. Ibid., *155:* 183.

SCHWEIGERT, B. S., I. E. TATMAN AND C. A. ELVEHJEM 1945 The leucine, valine and isoleucine content of meats. Arch. Biochem., *6:* 177.

STOKES, J. L., M. GUNNESS, I. M. DWYER AND M. C. CASWELL 1945 Microbiological methods for the determination of amino acids. II. A uniform assay for the 10 essential amino acids. J. Biol. Chem., *160:* 355.

TATUM, E. L., AND W. H. PETERSON 1935 Fermentation method for production of dextro-lactic acid. Ind. Eng. Chem., *27:* 1493.

HYPERVITAMINOSIS A IN THE RAT

KAARE RODAHL

Institute of Physiology, Oslo University, Oslo, Norway

EIGHTEEN FIGURES

(Received for publication January 9, 1950)

In a previous investigation (Rodahl, '49a, b) the toxic effect of polar bear liver was found to be due to its very high content of vitamin A, causing hypervitaminosis A in rats when ingested in large quantities.

This condition was first described by Takahashi et al. ('25) and has since been studied by many workers. Apart from symptoms such as emaciation, exophthalmos, soreness in the skin, and alopecia, the more specific lesions in hypervitaminosis A have been found to be spontaneous fractures of the bones (Collazo and Rodriguez, '33; Bomskov and Seeman, '33; and others) and hemorrhages (Rodahl and Moore, '43; Moore and Wang, '45; and others). Histologically, Drigalski and Laubmann ('33) found degeneration of the renal tubules and glomeruli, of the testes, cardiac muscle and, to a small extent, of hepatic cells. A number of investigators have detected rarefaction of the bones with extreme thinning of the cortex (Bomskov and Seeman, '33; Wolbach and Bessey, '42) and increased deposits of fat in the reticuloendothelial cells, especially the Kupffer cells of the liver and the spleen (Domagk and Von Dobeneck, '33; Collazo and Rodriguez, '33; Drigalski and Laubmann, '33; Uotila and Simola, '38; Noetzel, '39). Some authors have found evidence of changes in the internal secretory glands in hypervitaminosis A, such as increased activity of the thyroid gland (Uotila, '38) and hypertrophy of the island tissue of the pancreas

(Cornil, Chevallier and Paillas, '39). Bomskov and Sievers ('33) observed no effect on the formation of blood in hypervitaminotic rabbits, while the sedimentation rate was reduced, which has also been observed in rats (Rodahl, '49b). The serum calcium and phosphorus have been reported not to be affected (Bomskov and Seeman, '33). A temporary rise in cholesterol and in lipids of the serum following large doses of vitamin A has been reported by several workers (Wendt, '36; Josephs, '44), while a late drop in blood lipids has been observed in hypervitaminotic dogs (Maddock, Wolbach and Maddock, '49).

As an extension of previous work (Rodahl, '49b), the purpose of the present investigation was to examine in further detail the effect of an excess of vitamin A on rats, including a study of the relation between the symptoms and lesions in hypervitaminosis A.

EXPERIMENTAL

Experimental animals

For these experiments a total of 157 albino and piebald rats of both sexes and at different stages of development were used. All rats received daily as much as they wanted of a basal diet which had the following composition: oatmeal, 10.7%; corn meal, 19.5%; coarse wheat flour, 14.6%; wheat embryo, 9.8%; coarse rye flour, 14.6%; dried milk (skimmed), 23.4%; sodium chloride, 0.5%; calcium carbonate, 0.5%; dried yeast, 4.0%; and peanut oil, 2.4%. In addition to this, all rats in these experiments received 150 I.U. of vitamin A and 100 I.U. of vitamin D per rat once a week. Whole maize was given once or twice a week, and ordinary bread once a week. Fresh milk and water were given every day, and occasionally cabbage and carrots. Liver of pig, ox or calf was given once a week.

The rats were kept separated in special cages for each group, and all experimental animals lived under identical conditions. A number of experiments were carried out simultaneously, which allowed strict comparison.

Sources of vitamin A

Whale liver oil concentrate (200,000 I.U. vitamin A/gram) was used as the source of vitamin A in most of the experiments, since it had been found in previous work (Rodahl, '49b) that this source of vitamin A was readily consumed by the animals and contained no vitamin D. The possibility of introducing the condition of hypervitaminosis D is thus ruled out in the experiments where this source of vitamin A is used. In some cases vitamin A alcohol and acetate, obtained from fish oil concentrates, and in one case synthetic vitamin A acetate, were used for comparison. In one experiment rats were fed with the liver of a dog which had received huge doses of vitamin A concentrate during a period of 57 days prior to being killed, the liver containing 90,000 I.U. vitamin A/gram.

Arrangement of the experiment

In one experiment the effect of whale liver oil concentrate was compared with the effect of vitamin A alcohol and acetate. In a second experiment the effect of the various fractions of the whale liver oil were examined: the fraction containing the vitamin A, the oil after the vitamin A had been destroyed, the kitol, and the sterol fraction. In a third experiment the effect of excess vitamin A was compared when given to the rats mixed in the diet and when given by a catheter or pipet. In a 4th experiment the effect on rats of the subcutaneous injection of large doses of vitamin A was examined and compared with the effect of equivalent doses given orally. In a 5th experiment the effect of local application of vitamin A on the skin of rats was examined. In a 6th experiment the effect of single massive doses was compared with the effect of prolonged administration of an excess of vitamin A. In a 7th experiment the approximate gross dose of vitamin A sufficient to cause symptoms of hypervitaminosis A in young growing rats was determined by giving doses ranging from 47 to 625 I.U. vitamin A/gram body weight daily. In a final experiment the effect of an excess of vitamin A

on rats at various stages of development was examined in 5 groups of animals with initial body weights of from 21 to 246 gm.

The control groups were given either the basal diet only, or the vitamin A oil freed of its vitamin A content in addition to the basal diet. In some cases peanut oil was given to the control rats in the same quantities as the vitamin A oil was given to the experimental animals.

Clinical examination

Each experimental animal was carefully examined clinically every day. The animals were weighed every other day, and roentgenograms were taken of the long bones at intervals.

Post mortem examination

In all cases where the animals died the organs were examined as soon after death as possible. In all other cases they were anesthetized by ether for a few minutes to allow blood samples to be collected from living animals for examination, after which they were killed by decapitation and examined. Tissue sections were prepared of the liver, kidney, adrenals and other internal secretory glands, intestines, spleen, lungs, and heart, as well as the bones of both experimental and normal control animals. The following staining methods (Romeis, '24; Mallory, '38) were used: hematoxylin-eosin, van Gieson, Mallory, Kossa, Best's glycogen staining, and Turnbull's staining for blood pigment. Frozen sections of some of the internal organs, such as the liver, adrenals and in some cases the kindey, spleen, pancrease, intestines, lungs, heart and thymus gland, were stained by Sudan III. In the case of bones, the vacuum method described by Wærhaug ('49) was employed for decalcification, using formic acid (Lillie, '48).

When the weight of the adrenals was to be recorded, the organs were carefully removed immediately after death,

quickly placed in a small glass tube supplied with a cork, and weighed. In all cases the left adrenal was weighed and the tissue sections prepared from the other adrenal.

Blood examination

Hemoglobin was determined in all cases by the same Zeiss-Ikon hemoglobinometer (standard 13.8 gm Hb/100 ml blood = 100%). Blood counts were made in the usual manner. Vitamin A was determined in the serum by a modification of the usual antimony trichloride method (Rodahl, '50). For the determination of the total base, the electrodialysis method was used. Calcium was determined by the Kramer-Tisdall method (Kramer and Tisdall, '21) and phosphorus by Kuttner and Lichtenstein's method (Kuttner and Lichtenstein, '36). Blood sugar was determined by the Hagedorn-Jensen method (Hagedorn and Jensen, '23a, b) and the serum iron by Heilmeyer and Ploetner's method (Heilmeyer and Ploetner, '37), using *o*-phenanthroline hydrochloride as the indicator.

Urine examination

At intervals urine samples were collected during 24-hour periods from the various groups of rats by placing the animals in specially constructed cages where the urine was collected without being contaminated by feces. In some cases urine samples were also collected by puncture of the urinary bladder in ether anesthesia. The urine was examined with regard to the presence of protein, blood and sugar. In some cases Schlesinger's test was made.

Feces examination

At intervals feces were examined by the benzidine test in the usual manner. In some cases feces were collected during 24-hour periods, weighed and extracted by acetone in a Soxhlet's apparatus in a dark room; the vitamin A content was determined in the fat spectrographically and by the antimony trichloride method.

Determination of the ash and mineral content of the bones

The femur was removed immediately after death, cleaned and weighed. The bones were dried until a constant weight was reached at a temperature of 104°C. After this the bone was ashed at 800°C. until a constant weight resulted. The ash was dissolved in 1–n HCl and the calcium determined by the Kramer-Tisdall method. The phosphorus was determined by Kuttner and Lichtenstein's method.

RESULTS

Symptomatology

In agreement with previous findings (Rodahl, '49b, c), the symptoms following ingestion of toxic doses of vitamin A in rats may be divided into symptoms of acute intoxication and symptoms of chronic intoxication. Symptoms of acute intoxication may occur within a few hours after single toxic doses are given by pipet or catheter, in the form of general malady, changes in the pelts, drowsiness, muscular weakness and decreased activity. Simultaneously, a rapid increase of the vitamin A level in the blood was observed during the first 2 to 4 hours (up to 17 I.U. vitamin A/gram serum in rats given 50,000 to 90,000 I.U. vitamin A daily), followed by a rapid decrease. At the end of 48 hours the vitamin A level of the blood had usually reached normal levels and at the same time the animals given a single dose appeared to have recovered. If no further excess of vitamin A is given, it appears that the animal recovers without any detectable injurious effect. It is thus probable that the symptoms of acute intoxication are related to the sudden large increase of the vitamin A level of the blood.

Continued administration of toxic doses during a period of several days caused symptoms of chronic intoxication (fig. 1) such as: lack of appetite and reduced weight gain or loss of weight, muscular weakness, loss of hair (fig. 2), soreness and bleeding in the skin, swelling of the palpebrae,

exophthalmos, stiffness of the limbs, limping, spontaneous fractures, internal hemorrhages and eventually death. In some cases abnormal posture was observed, with marked bending of the spine and both hind legs appearing paralyzed, without any fractures or hemorrhages being detected in the lower extremities (fig. 3).

In hypervitaminotic rats there was a marked reduction of the food intake, which was found to be in proportion to the vitamin A doses given — that is the higher the dose of vitamin A, the lower the food intake. Thus, while normal control rats consumed an average of 14.5 gm food daily, rats of the same age given 10,000 I.U. vitamin A daily consumed 9.7 gm, those given 50,000 I.U. vitamin A daily consumed 5.9 gm, and animals given 60,000 I.U. vitamin A daily consumed only 3.6 gm food daily.

Examination of the urine usually revealed proteinuria and hematuria, and the amount of urine secreted in 24 hours was less in hypervitaminotic rats than in normal control rats. In the feces the benzidine test was often positive. In the majority of cases the hemoglobin values fell within the range of those of the normal control rats. Reduced hemoglobin values were sometimes associated with considerable hemorrhages, which may possibly explain the anemia in those cases. The incidence of spontaneous macroscopical hemorrhages in hypervitaminotic rats was lower in the present experiments than has generally been accepted. Out of 62 rats, spontaneous hemorrhages occurred in 14 cases; of these, 7 occurred in young rats, and 7 in adult animals. A considerable reduction of the serum iron was detected in only one rat (35 μg%), in which case a marked anemia was also observed (Hb: 65%). No significant difference was detected in the differential blood count in hypervitaminotic rats as compared with that of the normal control rats. The sedimentation rate was reduced to 1 to 2 mm after one hour in hypervitaminotic rats, as against the usual 5 to 10 mm in normal rats. The levels of total base, potassium, urea, calcium and phosphorus in the serum were within the normal

range. The fasting blood sugar was normal in all cases except three. In one rat, which was moribund, it was only 60 mg/100 ml, and in two cases it was 165 mg/100 ml.

The average glycogen content of the liver was 70 mg/100 gm in hypervitaminotic rats as against 110 in normal rats. In 6 cases it was abnormally low in the first-mentioned group (10 to 20 mg/100 gm). This was probably due to inanition.

No direct relation could be detected between the severity of the clinical symptoms in rats given prolonged administration of an excess of vitamin A and the vitamin A level in the blood. Thus, the vitamin A level of the blood 24 hours after the last dose of vitamin A was 2.8 to 4.9 I.U./gram both in rats receiving doses of 10,000 I.U./day — which produced practically no clinical symptoms — and in rats given toxic doses of between 50,000 and 90,000 I.U. vitamin A daily and which showed pronounced clinical symptoms.

The vitamin A content was largely increased in all the internal organs examined in hypervitaminotic rats, such as the liver, kidney, adrenals, and testes, as well as the visceral fat. In none of the cases was any vitamin A detected in the urine from hypervitaminotic rats, although the vitamin A content of the kidney was as high as 1,800 I.U. vitamin A/gram. No direct relation could be detected between the vitamin A content of the rat's liver and the manifestation of clinical symptoms of hypervitaminosis A. On the other hand, it appears that the most significant pathological changes are detected in the organs of hypervitaminotic animals where the storage of vitamin A is highest, such as in the liver and kidneys.

Systematic x-ray examination of the long bones at various stages of the experiment showed gradual thinning of the shaft. In agreement with this, it was found that in both young and adult rats the diameter of the femur was less in hypervitaminotic animals than in normal control rats when bones of the same length were compared in the two cases and the diameter was measured at corresponding places at the post mortem (fig. 4). The bones appeared normally calcified,

but lines of denser calcification were seen in the epiphyses as well as in the epiphyseal border of the metaphyses. The cortical shadow was abnormally thin, particularly toward the metaphyses, where it was usually absent. Simultaneously it was observed that the epiphyseal line became abnormally narrow as compared with that in normal rats of the same age. In very young rats roentgenograms also showed a broad dense shadow around the centre of ossification, an irregularly broadened intensely calcified zone at the epiphyseal ends of the tibia, and a glass transparency of the shaft. Healing of the fractures took place at the same rate as healing of fractures produced artificially in normal rats for comparison, in spite of the vitamin A's being given continuously in unchanged doses, although abnormally rich callus formation was usually observed in the former (figs. 5, 6, 7, 8).

Macroscopic post mortem examination in many cases revealed no significant pathological findings. The most constant findings were emaciation; arterial hyperemia; subcutaneous, muscular or visceral hemorrhages; fractures; enlarged adrenals (the weight of the adrenals when expressed as per cent of the body weight of the animal was up to three times higher in hypervitaminotic rats than in normal control rats of the same age); fatty appearance of the cut surface of the liver; swelling of the visceral lymph glands; and widening of the costochondral junction in some cases. In some of the rats the lungs and other internal organs were fiery red.

By microscopic examination the most constant findings were hyperemia; scattered red blood cells outside the capillaries in the internal organs, in the space of Bowman's capsule (fig. 11), and in the tubular lumen of the kidney (fig. 12); degeneration of the renal tubules (fig. 10); deposits of sudanophil droplets between the liver cells, particularly in the Kupffer cells which appeared swollen; and slight fatty degeneration of the actual liver cells (fig. 9). By Sudan staining large deposits of sudanophil droplets were usually seen in the adrenal cortex (figs. 17, 18). Histological examination of pieces of the skin taken from areas with marked alopecia

revealed hyperemia and massive hemorrhages around the root sheaths of the hair (fig. 13). This may probably be considered the cause of the alopecia. Microscopical examination of the long bones revealed marked hyperemia and subperiosteal and osseous hemorrhages, with destruction of bone spicules, an increased number of osteoclasts, and thinning of the compact bone (figs. 15, 16).

Chemical analysis of the femurs in young and adult hypervitaminotic and in normal control rats showed no significant reduction in the calcium or phosphorus content of the ash in the former animals as compared with normal rats, while the ash content of the dry bone in some cases was reduced and generally showed considerable variation among animals.

Identical effects were obtained in rats by feeding mammalian livers rich in vitamin A — such as the liver of a hypervitaminotic dog, liver oils, highly purified vitamin A concentrates, and synthetic vitamin A acetate — when these were given orally in equivalent amounts with regard to the vitamin A content. No ill effect whatever was observed in rats given liver oil where the vitamin A had been destroyed. None of the fractions of the liver oils other than the vitamin A had any ill effect on rats.

The same toxic effect was observed when an excess of vitamin A was administered orally or given by subcutaneous injection. The quantities of vitamin A oils required to give rise to toxic symptoms were considerably higher, however, when injected subcutaneously than when given orally. This is probably due to incomplete and slow absorption of the vitamin A from the subcutaneous tissue. On the other hand, vitamin A emulsions were readily absorbed following injections, the effect of equivalent doses of vitamin A in this form being identical when compared with oral administration. Local application of vitamin A concentrates to the skin of rats caused no ill effect.

Subcutaneous injection of single massive doses of vitamin A into rats has been found by some previous workers (Takahashi et al., '25; Matsuoka, '34) to cause death from cramps.

In none of our experiments did cramps occur as a result of the subcutaneous injection of large quantities of vitamin A oils — up to one million I.U. of vitamin A in a single injection — when great care was taken not to introduce the oil into the blood stream.

Identical gross doses of vitamin A oils appeared to prove slightly more injurious to rats when partly mixed in the basal diet than when given entirely by pipet or by a specially constructed catheter. In the latter case a larger proportion — approximately 20% more — of the gross dose of vitamin A was lost through the feces, and it is possible that this may account for the difference in the observed effect.

Oral administration of single massive doses of vitamin A, apart from causing symptoms of acute intoxication shortly after the dose, had only a slight delayed effect on rats, and produced no detectable pathological changes in the internal organs and bones. In no case was it possible to produce lethal effects by single doses of vitamin A, such as 1.6 million I.U. of the vitamin given in the course of three hours. Nor did a very high vitamin A level in the blood (31 I.U. vitamin A/gram serum) in itself prove lethal.

It is thus evident that the prolonged administration of an excess of vitamin A in toxic doses over a period of several days is necessary to produce the changes characteristic of the condition called hypervitaminosis A.

Toxic dose

Rodahl and Moore ('43), and Herbst, Pavcek and Elvehjem ('44) found that doses of over 15,000 I.U. vitamin A daily are toxic to rats. This has been confirmed in the present investigation. An apparent effect of age on the manifestations of hypervitaminosis A in rats given similar gross doses of vitamin A has previously been reported by Rodahl and Moore ('43); Moore and Wang ('45); and Walker, Eylenburg and Moore ('47). On the basis of the present experiments, however, it appears that under identical conditions equivalent daily doses of vitamin A produce the same clinical picture

and post mortem findings in young, adolescent and adult rats. Contrary to the findings previously reported, there was in these experiments no higher incidence of hemorrhages in adult rats than in young rats and the same bone changes occurred, with a similar frequency of spontaneous fractures, although the fractures developed later in adult than in young growing animals.

Confirming the statement made by Rodahl and Moore ('43), the clinical picture of hypervitaminosis A was found to vary remarkably with the magnitude and duration of the overdosage. In all cases daily doses of over 50 to 100 I.U. vitamin A/gram of body weight resulted in appreciable toxic manifestations in rats. Doses of between 47 and 135 I.U. vitamin A/gram of body weight produced only slight toxic symptoms, with moderately reduced weight gain and slight proteinuria and hematuria. Apart from these, no other clinical symptoms were observed even when this dose was given daily during a period of 58 days. In all cases, doses of between 200 and 500 I.U. vitamin A/gram of body weight caused pronounced clinical symptoms and pathological changes typical of hypervitaminosis A. Doses of over 800 I.U. vitamin A/gram of body weight proved lethal in all cases at the end of periods varying from 9 to 30 days. These figures must be taken as maximum values, however, since it has been shown in experiments with rats that approximately 40% of the ingested amount of vitamin A is lost through the feces.

DISCUSSION

It is a question whether all of the observed symptoms may be considered as directly caused by an excess of vitamin A as such, or whether some of them may be indirect rather than direct results.

Absence of normal weight gain is one of the first and most constant symptoms in hypervitaminotic rats. This was found to be associated with reduced food intake as a result of lack of appetite. This symptom must be considered as being attributable to the action of an excess of vitamin A, since the

oil had no adverse effect on the appetite or the weight gain when the vitamin A had been destroyed.

It would seem reasonable to assume that inanition in the most pronounced cases may have been in part responsible for some of the symptoms of hypervitaminosis A. On the other hand, a number of these symptoms were observed in direct connection with the first massive doses of vitamin A, before the effect of inanition would be noticeable. The importance of this factor, however, should be studied further by limiting the food intake of the control rats to that of the test animals.

As judged by roentgenograms and microscopical examination, it appeared that destruction and absorption of the bone substance occurred in hypervitaminotic rats, while the remaining bone, although irregular in structure, appeared to contain normal richness of calcium and normal density of bone cells. This may well explain the fact that the calcium and phosphorus contents of the ash in the remaining bone appeared unaltered in hypervitaminotic animals.

Wolbach ('46) considers that bone remodelling is greatly accelerated in hypervitaminosis A, and that a defective tissue is laid down which fractures easily. On the other hand, it seems probable that at least some of the observed bone changes may be explained as the result of the hemorrhages, particularly those which occur around the periosteal blood vessels, and of lack of nutrition caused by insufficient blood supply. In fact, a combination of several factors may be the most likely explanation of the tendency to spontaneous fractures in hypervitaminosis A.

In most cases the fractures occurred near the end of the long bones, at the places where the compact bone was thinnest and where it would be expected to offer less resistance against breakage. In some cases, however, fractures occurred at places where the compact bone was thicker, thus indicating an abnormal fragility of the bones (fig. 14).

The tendency to bleeding (hemorrhagic diathesis) is a prominent feature in the clinical and pathological picture of

hypervitaminosis A. The hemorrhages appeared to be quite general — subcutaneous, muscular and visceral — and varied from small petechia in the skin, or scattered red blood cells outside the capillaries in the internal organs, to massive blood extravasations and the formation of large hematomas. Epistaxis and bleeding from the gums were also observed. Hemorrhages often occurred in places where no serious injury was likely to start the bleeding, and an additional supply of vitamin K (Rodahl, '49b) had no beneficial effect on them.

Histologically, scattered red blood cells were seen outside the capillaries in the internal organs such as the liver, kidney and lungs, and in some cases also the adrenals, as well as in the bones, indicating an abnormal permeability of the capillary wall for the red blood cells. By the usual staining methods no morphological abnormalities were detected in the vessel walls, however.

In the kidneys, free blood was frequently observed in the tubules and in the space of Bowman's capsule, indicating an abnormal permeability of the capsule for red blood cells. This may well explain the hematuria which was a frequent finding in hypervitaminotic rats and which was sometimes very marked.

The condition of hypervitaminosis A presents a highly complex clinical picture. Several of the clinical symptoms have been found to correspond to histological changes attributed to excess of vitamin A. Nevertheless, the mechanism of the toxicity is still, on the whole, quite obscure.

SUMMARY AND CONCLUSIONS

As an extension of previous investigations (Rodahl, '49b), the effect of an excess of vitamin A on rats has been studied in further detail.

Oral administration of an excess of vitamin A produced the same toxic symptoms as subcutaneous injection.

Prolonged administration of an excess of vitamin A in toxic doses over a period of several days was necessary to

produce the changes characteristic of the condition called hypervitaminosis A.

No significant effect of age or sex was observed on the clinical picture of hypervitaminosis A in rats given equivalent daily doses of the vitamin, per gram of body weight.

Gross doses of over 50 to 100 I.U. vitamin A/gram of body weight daily resulted in appreciable toxic manifestations. While doses of between 47 and 135 I.U. vitamin A/gram of body weight produced only slight toxic symptoms, doses of between 200 and 500 I.U. caused pronounced clinical symptoms, and doses over 800 I.U./gram of body weight proved lethal in all cases at the end of periods varying from 9 to 30 days.

The symptoms following ingestion of toxic doses of vitamin A were: changes in the pelts, drowsiness, muscular weakness, lack of appetite, reduced growth, soreness and bleeding of the skin, alopecia, swelling of the palpebrae, exophthalmos, stiffness in the limbs, limping, spontaneous fractures, hemorrhages, proteinuria, hematuria, intestinal bleeding, and in some cases hypochromic or normochromic anemia. The fasting blood sugar was reduced in one case and increased in two cases. No abnormalities were detected in the content of calcium, phosphorus, total base, potassium and urea in the blood, and no significant difference was detected in the differential blood count when compared with that of normal rats.

X-ray and microscopic examination of the long bones revealed characteristic changes, while no significant changes were detected in the mineral content of the bones.

Histologically, the most constant findings were hyperemia, scattered red blood cells outside the capillaries in the internal organs and in the space of Bowman's capsule in the kidney, degeneration of the renal tubules, deposits of sudanophil droplets in the liver and increased sudanophil deposits in the adrenal cortex.

Several of the clinical symptoms in hypervitaminosis A were found to correspond to histological changes attributed to the toxic effect of an excess of vitamin A. The mechanism of this toxic effect on the cells is, however, unknown.

ACKNOWLEDGMENTS

The present work has been carried out at the Institute of Physiology, Oslo University, Oslo, Norway. I am greatly indebted to the head of this institute, Professor Einar Langfeldt, for placing the necessary laboratory facilities at my disposal. The work has been carried out during a full-time grant from Norsk Polarinstitutt, for which I express my sincere thanks. My thanks are also due to the head of that institute, Professor H. U. Sverdrup, for his interest and help in the completion of this work. I am also indebted to Distillation Products, Inc., Rochester, N. Y., for furnishing the synthetic vitamin A acetate.

LITERATURE CITED

BOMSKOV, C., AND G. SEEMAN 1933 Über eine Wirkung des Vitamin A auf den Mineralhaushalt. Zschr. ges. exp. Med., *89:* 771.

BOMSKOV, C., AND G. SIEVERS 1933 Über die Wirkung des A-Vitamine auf die Zusammensetzung des Blutes. Ibid., *89:* 780.

COLLAZO, J. A., AND J. S. RODRIGUEZ 1933 Hypervitaminose A. I. and II. Klin. Wschr., *12:* 1732, 1768.

CORNIL, L., A. CHEVALLIER AND J. E. PAILLAS 1939 Histologic study of viscera in experimental hypervitaminosis A in the guinea pig. Ann. d'anat. path., *16:* 74.

DOMAGK, G., AND P. VON DOBENECK 1933 Über histologische Befunde bei der Überdosierung mit Vitamin A-konzentrat. Arch. path. Anat., *290:* 385.

DRIGALSKI, W., AND W. LAUBMANN 1933 Über Schadigung durch Vitamin A. Klin. Wschr., *12:* 1171.

HAGEDORN, H. C., AND B. N. JENSEN 1923a Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. Biochem. Zschr., *135:* 46.

———— 1923b Die Ferricyanidmethode zur Blutzuckerbestimmung. II. Ibid., *137:* 92.

HEILMEYER, L., AND K. PLOETNER 1937 Das Serumeisen und die Eisenmangelkrankheil. Fisher, Jena.

HERBST, E. J., P. L. PAVCEK AND C. A. ELVEHJEM 1944 Telang livers and vitamin A toxicity. Science, *100:* 338.

JOSEPHS, H. W. 1944 Hypervitaminosis A and carotenemia. Am. J. Dis. Child., *67:* 33.

KRAMER, B., AND F. F. TISDALL 1921 A simple technique for the determination of calcium and magnesium in small amounts of serum. J. Biol. Chem., *47:* 475.

KUTTNER, T., AND L. LICHTENSTEIN 1936 Micro colorimetric studies. Ibid., *85:* 671.

LILLIE, R. D. 1948 Histopathologic Technic. The Blakiston Company, Philadelphia and Toronto.

MADDOCK, C. L., S. B. WOLBACH AND S. MADDOCK 1949 Hypervitaminosis A in the dog. J. Nutrition, *39:* 117.

MALLORY, F. B. 1938 Pathological Technique. W. B. Saunders Company, Philadelphia and London.

MATSUOKA, M. 1934 On cramps caused by an over-dose injection of biosterol. Bull. Inst. Phys. Chem. Res., Japan, *13:* 1267 (English abstract).

MOORE, T., AND Y. L. WANG 1945 Hypervitaminosis A. Biochem. J., *39:* 222.

NOETZEL, H. 1939 Morphologische Untersuchungen bei Vitamin A — überdosierung. Zschr. ges. exp. Med., *105:* 83.

RODAHL, K. 1949a The toxicity of polar bear liver. Nature, *164:* 530.

———— 1949b The toxic effect of polar bear liver. Norsk Polarinstitutts Skrifter, no. 92.

———— 1949c Hypervitaminosis A and scurvy. Nature, *164:* 531.

———— 1950 Hypervitaminosis A. Norsk Polarinstitutts Skrifter, no. 95.

RODAHL, K., AND T. MOORE 1943 The vitamin A content and toxicity of bear and seal liver. Biochem. J., *37:* 166.

ROMEIS, B. 1924 Taschenbuch der Mikroskopischen Technik. München and Berlin.

TAKAHASHI, K., Z. NAKAMIYA, K. KAWAKIMI AND T. KITASATO 1925 On the physical and chemical properties of biosterin and on its physiological significance. Sci. Pap., Inst. Phys. Chem. Res., Tokyo, *3:* 81 (English abstract).

UOTILA, U. 1938 Über die Schilddrüsenveränderungen bei A-, B-, und D-Avitaminosen. Arch. path. Anat., *301:* 535.

UOTILA, U., AND P. E. SIMOLA 1938 Über die Beziehungen zwischen den Vitaminen und dem reticuloendothelialen System. Ibid., *301:* 523.

WAERHAUG, J. 1949 Decalcification of bone and teeth under vacuum — a rapid method for producing hard tissue preparations. J. Dent. Res., *28:* 525.

WALKER, S. E., E. EYLENBURG AND T. MOORE 1947 The action of vitamin K in hypervitaminosis A. Biochem. J., *41:* 575.

WENDT, H. 1936 Hypercholesterinämie und Vitamin A. Deut. med. Wschr., *2:* 1213.

WOLBACH, S. B. 1946 Vitamin A deficiency and excess in relation to skeletal growth. Proc. Inst. Med., Chicago, *16* (4).

WOLBACH, S. B., AND O. A. BESSEY 1942 Tissue changes in vitamin deficiencies. Physiol. Rev., *22:* 233.

PLATE 1

EXPLANATION OF FIGURES

1 Young rat (initial body weight 42 gm) given 830–1300 I.U. vitamin A/gram body weight daily for 18 days, showing multiple fractures and scruffiness, the hair being smeared with urine and feces.

2 Alopecia and soreness of left eye, which is completely closed, in young rat given 4 drops daily of a highly concentrated vitamin A preparation, corresponding to 60,000 I.U. vitamin A daily (697 I.U. vitamin A/gram body weight).

3 Abnormal posture, with marked bending of the spine, and "paralysis" of both hind legs, without any fracture or hemorrhages being detected in the lower extremities in adult rat given 625 I.U. vitamin A/gram body weight daily for 24 days.

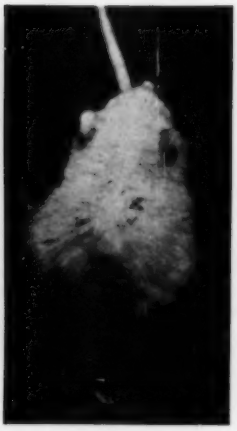
4 Femur of adult hypervitaminotic rat (left), compared with the femur of a normal control rat of the same age (right), showing the difference in diameter.

5, 6 and 7 Roentgenograms of right hind leg of female rat given 50,000 I.U. vitamin A daily (500–250 I.U. vitamin A/gram body weight) at the end of 53 days (5), showing abnormal thinning of the bone shafts, and at the end of 74 (6) and 106 days (7), showing healing of fractures of the tibia and fibula.

8 Healing of fractures in hypervitaminotic rat with marked callus formation.

1

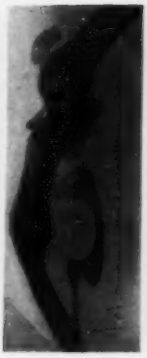
2



3

4

5



6

7

8

PLATE 2

EXPLANATION OF FIGURES

9 Photomicrograph (× 150). Liver, stained by Sudan III, from adult male rat given 230–390 I.U. vitamin A/gram body weight daily, showing massive deposits of sudanophil droplets between the liver cells, especially in the Kupffer cells.

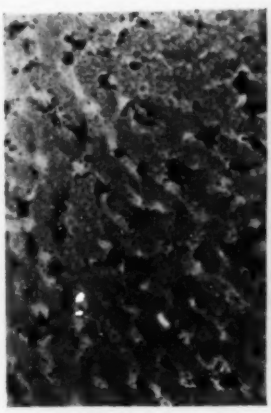
10 Photomicrograph (× 285. Hematoxylin-eosin stain). Kidney from rat given 500–250 I.U. vitamin A/gram body weight daily for 119 days, showing degeneration and necrosis of convoluted tubules.

11 Photomicrograph (× 375. Mallory stain). Kidney from rat given 52,500 I.U. vitamin A daily for 11 days, showing red blood cells in the space of Bowman's capsule.

12 Photomicrograph (× 335. Hematoxylin-eosin stain). Kidney from same rat shown in figure 11, showing red blood cells in collecting tubules.

13 Photomicrograph (× 60. Hematoxylin-eosin stain) of slide taken from the skin at the place of alopecia in a rat given 52,500 I.U. vitamin A daily for 11 days, showing marked hyperemia and hemorrhages around the root sheaths of the hair (in the dermic coat).

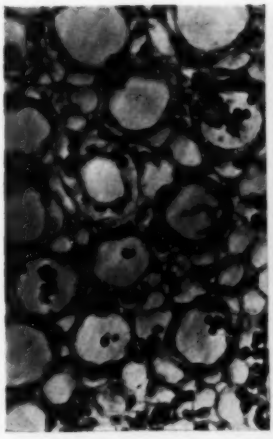
14 Photomicrograph (hematoxylin-eosin stain). Fracture of tibia in rat given excess of vitamin A (570 I.U. vitamin A/gram body weight daily) for 21 days.



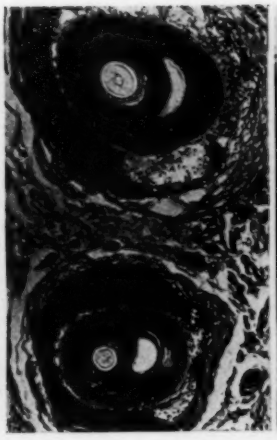
9

10

11



12



13

14

PLATE 3

EXPLANATION OF FIGURES

15 Photomicrograph (× 22. Hematoxylin-eosin stain). Tibia from young rat given 95 I.U. vitamin A/gram body weight daily for 18 days, showing no significant pathological conditions.

16 Photomicrograph (× 22. Hematoxylin-eosin stain). Tibia from rat of same age as animal in figure 15, given 500–830 I.U. vitamin A/gram body weight daily for 27 days, showing narrow epiphyseal line, thinning and irregularity of compact bone and subperiosteal hemorrhage.

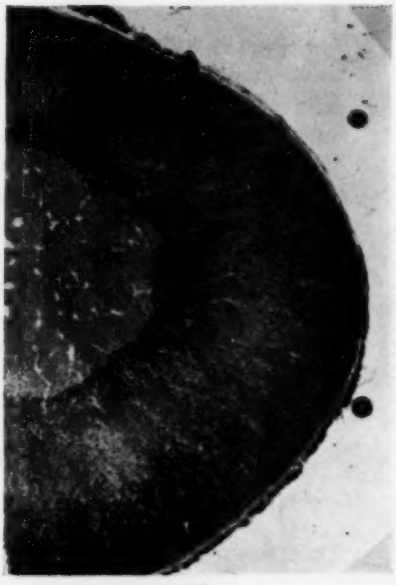
17 Photomicrograph (× 24). Adrenal of normal adult female rat stained by Sudan III.

18 Photomicrograph (× 24). Adrenal of adult male hypervitaminotic rat (250–390 I.U. vitamin A/gram body weight daily for 46 days) stained by Sudan III, showing dense sudanophil deposits in the cortex.

15

16

17



18

CONGENITAL ANOMALIES IN THE CHICK DUE TO VITAMIN B_{12} DEFICIENCY [1, 2, 3, 4, 5]

ORLANDO OLCESE, J. R. COUCH, J. H. QUISENBERRY AND P. B. PEARSON

Departments of Biochemistry and Nutrition and Poultry Husbandry, A. and M. College of Texas and Texas Agricultural Experiment Station, College Station

TWO FIGURES

(Received for publication February 6, 1950)

It is recognized that several nutritional deficiencies affect embryonic development in the chick. Chondrodystrophy, syndactyly, crooked tibia, perosis and other pathological conditions have been reported to occur in embryos as a consequence of nutritional deficiencies (Cravens, '49). While it is recognized that folic acid and biotin deficiencies produce perotic chicks and embryos (Daniel et al., '46; Richardson et al., '42; Cravens et al., '44; Couch et al., '48), it has also been reported that eggs from hens fed high amounts of soybean oil meal produce perotic chicks (Carver et al., '47). This perosis apparently is not related to folic acid or biotin deficiency and its incidence is decreased by the addition of fish meal to the ration. Similar results have been reported by

[1] Supported in part by a grant from The Lederle Laboratories, Pearl River, New York.

[2] The crystalline vitamins and APF concentrates used in this study were generously supplied by Merck and Company, Inc., Rahway, N. J., and the Lederle Laboratories, Pearl River, New York.

[3] Soybean protein was supplied through the courtesy of the Buckeye Cotton Oil Company, Cincinnati, Ohio.

[4] Soybean oil was contributed by Dr. J. E. Hunter, Allied Mills, Inc., Libertyville, Illinois, and the Buckeye Cotton Oil Company, Cincinnati, Ohio.

[5] The methionine and glycine used in this study were supplied through the courtesy of Dr. Julius Johnson, Dow Chemical Company, Midland, Michigan.

Bird et al. ('46), who found perosis in chicks fed high levels of soybean oil meal. In addition, these workers reported a lack of development of the muscles of the leg in embryos coming from hens fed the high soybean oil meal diet. In the light of the most recent investigations in connection with the animal protein factor (APF) and vitamin B_{12}, it is possible that a lack of this factor may have been responsible for the perosis observed by these two groups of investigators. Recently Olcese, Couch and Lyman ('49b) have shown that vitamin B_{12} is necessary for normal hatchability in the mature domestic fowl.

The present study is a report of observations made in the course of two experiments to determine the gross pathological symptoms exhibited by embryos coming from hens fed a vitamin B_{12}-deficient diet.

EXPERIMENTAL PROCEDURE

Two experiments were run, with three groups of 4 Single Comb White Leghorn hens in each test. The birds were kept in individual laying cages and were artificially inseminated weekly with mixed semen from New Hampshire cockerels. The eggs were collected daily and marked with the hen's number and date. Settings were made weekly. The eggs were candled on the 7th and 18th days of incubation and at that time the infertile eggs and eggs containing dead embryos were removed and broken. The time of death of these embryos, as well as of those that failed to hatch on the 22nd day of incubation, was estimated and the embryos were carefully examined for gross pathological symptoms.

The composition of the diets used in this study is shown in table 1. In the first experiment diets A_1, A_3 and A_4 were used with APF concentrate R-1 (Lederle) as the source of vitamin B_{12}. Diet A_1 was the basal vitamin B_{12}-deficient diet. In diets A_3 and A_4 the basal diet was supplemented with 5 and 10 gm of APF concentrate R-1 (Lederle) per kilogram of diet, respectively. In the second experiment diets A_1, A_{11} and A_{12} were used with APF concentrate 1 (Merck) as the

source of vitamin B_{12}. The groups fed diets A_{11} and A_{12} received a supplement of 5 and 10 gm of APF concentrate 1 per kilogram of diet, respectively. Oyster shell and tap water were supplied to all hens ad libitum.

TABLE 1

Composition of diets[1]

CONSTITUENTS	DIET				
	A_1	A_2	A_4	A_{11}	A_{12}
	%	%	%	%	%
Sucrose	68	68	68	68	68
Soybean protein	22	22	22	22	22
Soybean oil	3	3	3	3	3
A and D oil (3000A–400D)	2	2	2	2	2
Salts IV	5	5	5	5	5
	Supplement (gm/kg)				
APF conc. R-1 (Lederle)	0	5	10		
APF conc. no. 1 (Merck)	0			5	10

[1] The following amounts of vitamins and amino acids were added per kilogram of diet: Thiamine hydrochloride 4 mg, riboflavin 6 mg, calcium pantothenate 15 mg, niacin 100 mg, pyridoxine hydrochloride 4 mg, 2-methyl-1,4-naphthoquinone 0.5 mg, alpha-tocopherol 3 mg, pteroylglutamic acid 2 mg, biotin 0.2 mg, choline chloride 2,000 mg, DL-methionine 7.5 gm, and glycine 4 gm.

RESULTS AND DISCUSSION

Age of the embryos at time of death

During the course of the two experiments conducted in this study, a peak of embryonic mortality was found at about 16 to 18 days of incubation in eggs laid by hens fed the basal diet low in vitamin B_{12}. In figure 1 the per cent mortality in the whole experimental period of the groups fed the low-vitamin B_{12} diets has been plotted against the age of the embryos at the time of death. In the first experiment 29% of the deaths of embryos occurred at the 17th day of incubation, and a total of 53% of the deaths occurred between the 16th and the 18th day. In the second experiment, 40% of the embryonic deaths occurred at the 17th day and 50% of the total deaths occurred between the 16th and 18th days of incubation.

A slight rise in mortality was also found toward the last day of incubation in both experiments. On the other hand, the number of embryos that died near the 17th day of incubation was decreased when the diet was supplemented with a source of vitamin B_{12} (groups 2, 3, 5 and 6, table 2).

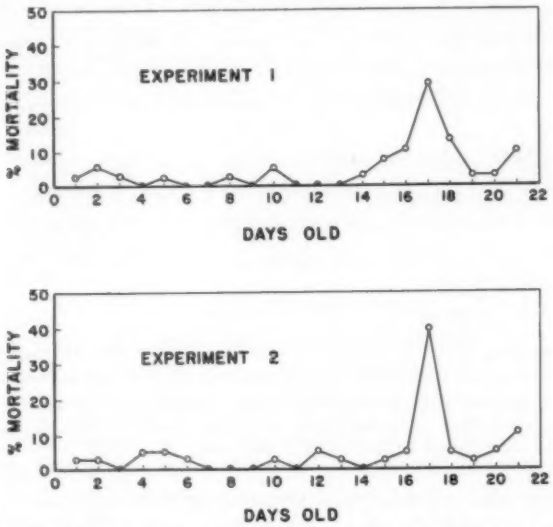


Fig. 1 Age of embryos at time of death in eggs from hens fed diet A_1 (low vitamin B_{12}).

Embryonic anomalies

The distribution of gross pathological symptoms in embryos from the different groups of pullets is shown in table 2. The most characteristic deformity under conditions of vitamin B_{12} deficiency consisted of the presence of spindly legs, a condition which is designated as "myoatrophy of the leg" in the present report.

Myoatrophy of the leg. This condition is characterized by the presence of very slender legs which are about normal in length. The legs are abnormally red in color. The latter is probably due to a general hemorrhagic condition. This color-

ation is shown not only by the legs but is also extended to the body. The hemorrhages are clearly seen in the muscles and also in the cartilaginous portions of the skeleton. As a consequence, the skeletal articulations showed a dark color which was especially apparent in the tibiotarsal-tarsometatarsal joint. Embryos which exhibited this condition died near the 17th day of incubation. Embryos which show very clearly the condition of myoatrophy of the leg can be seen in figure 2, where two abnormal embryos are shown, together with a

TABLE 2

Classification of embryonic abnormalities in hens fed different amounts of APF concentrates

GROUP	GRAMS OF APF SUPPLEMENT PER KILOGRAM OF DIET	FERTILE EGGS	EMBRYONIC MORTALITY			INCIDENCE OF MYOATROPHY OF THE LEG	POSITION OF EMBRYO IN EGG		HEMORRHAGES	
			1–7 days	6–17 days	18–21 days		Normal	Head between thighs	In body	In allantois
1	0	53	6	28	7	18	12	21	14	2
2	5 (Lederle R-1)	88	8	20	9	9	18	11	4	5
3	10 (Lederle R-1)	86	6	1	16	0	14	3	0	0
4	0	63	8	28	10	22	10	22	18	0
5	5 (Merck no. 1)	72	30	5	14	0	17	1	2	1
6	10 (Merck no. 1)	68	18	2	10	0	11	1	0	0

normal one for comparison. In certain cases hemorrhages in the allantois were present along with myoatrophy of the leg.

The incidence of myoatrophy of the leg in the two experiments in this study was very high in the two groups of pullets fed the basal ration deficient in vitamin B_{12} (groups 1 and 4). On the other hand, when the APF concentrate R-1 (Lederle) supplement was fed at a level of 5 gm per kilogram the incidence of myoatrophy of the leg decreased to about one-half that in the unsupplemented group. By increasing the level of this same supplement to 10 gm per kilogram, the incidence of this abnormality was reduced to zero. The ad-

dition of the APF concentrate 1 (Merck) supplement to the basal ration at levels of either 5 gm or 10 gm per kilogram reduced the incidence of myoatrophy of the leg to zero.

Anomalies of the embryo in the egg. The feeding to hens of the basal diet deficient in vitamin B_{12} resulted in a high incidence of embryonic malpositions in the egg (table 2). The most common malposition found was that of "head between the thighs." This anomaly is the same as that of Landauer's malposition I ('48). The incidence of this malposition decreased to about half when the hens received 5 gm

Fig. 2 Embryos with myoatrophy (left and right) and normal embryo (center). Removed from eggs 18th day of incubation.

of APF concentrate R-1 (Lederle) per kilogram of diet. A further decrease was observed when the diet was supplemented with 10 gm of this concentrate per kilogram of ration. When the APF concentrate 1 (Merck) was added to the basal ration, the incidence of malposition I decreased to only one embryo in each case, i.e., when two groups of hens received 5 and 10 gm of the concentrate per kilogram of diet, respectively.

Hemorrhages. The presence of hemorrhages was frequently noticed in embryos from the groups fed the basal diet. In this instance again the addition of 10 gm of APF concentrate R-1 (Lederle) gave complete protection against hemorrhages either in the body or in the allantois. In the same

way, 10 gm of APF concentrate 1 (Merck) afforded complete protection against hemorrhages. With this same concentrate fed at the level of 5 gm per kilogram of diet, the incidence of hemorrhages was greatly reduced.

In general the APF concentrate 1 (Merck) was more effective for prevention of all the symptoms of vitamin B_{12} deficiency reported above than was APF concentrate R-1 (Lederle), since at the level of 5 gm per kilogram the former concentrate afforded about the same protection as did the latter at the level of 10 gm per kilogram. When these two concentrates were assayed for vitamin B_{12} by a microbiological method, using *L. leichmannii* (ATCC 4797) as the test organism (Skeggs et al., '48), it was found that APF concentrate R-1 (Lederle) contained about 1 μg of apparent vitamin B_{12} per gram and that APF concentrate 1 (Merck) contained about 4 μg of apparent vitamin B_{12} per gram. On the basis of the above microbiological assay, the feeding of 10 gm of concentrate R-1 per kilogram of diet would supply 10 μg of vitamin B_{12} per kilogram of diet. This amount seems to afford complete protection against the described symptoms of vitamin B_{12} deficiency. The possibility exists, however, that a certain amount of vitamin B_{12} may not be readily available for microbiological determination with *L. leichmannii* 4797. At the level of 5 gm per kilogram of diet, APF concentrate 1 (Merck) was contributing 20 μg of vitamin B_{12} per kilogram of diet. In this case no incidence of myoatrophy of the leg, the most characteristic symptom of vitamin B_{12} deficiency, was noted. From these data it may be concluded that the minimum requirement of the laying hen for vitamin B_{12} is between 10 and 20 μg per kilogram of diet.

The mortality of the embryos in eggs from the groups receiving the APF concentrates was low at the 17th day of incubation with the exception of group 2 (table 2), which received 5 gm of APF concentrate R-1 (Lederle) per kilogram. This apparently was a suboptimum level of vitamin B_{12}. A certain degree of mortality was found, however, at other times of the incubation period in groups 2, 3, 5 and 6 (table 2).

This mortality was higher in groups 5 and 6, which were fed APF concentrate 1 (Merck), than in those fed APF concentrate R-1 (groups 2 and 3), even though the former concentrate was higher in vitamin B_{12} content. Apparently the deficiency of another factor is responsible for this mortality. Recently, Olcese, Couch and Lyman ('50) have shown that the addition of vitamin B_{12} concentrates to a sucrose-soybean diet similar to the one used in this study does not bring about normal hatchability in the mature domestic fowl. Olcese and Couch ('49a) have also found that the injection of crystalline vitamin B_{12} into eggs from hens fed the same vitamin B_{12}-deficient diet used in this experiment produces normal chicks up to the 9th experimental week. After this time the injection of vitamin B_{12} into such depleted eggs failed to restore normal hatchability. However, injection of these eggs with vitamin B_{12} prevented the symptoms of vitamin B_{12} deficiency associated with embryonic development, which are described in the present communication. Since the experiments reported in this study were conducted for 9 and 10 weeks, respectively, it is obvious that the symptoms observed and described herein were due to vitamin B_{12} deficiency alone.

Perosis. During the 8th experimental week two chicks were hatched from one of the hens receiving the basal diet. These chicks showed a very bad perosis in both legs and were unable to stand. The tissues of one chick were cleared according to the procedure of Couch et al. ('48), but no evidence of bone malformation was apparent.

It is possible that a small incidence of perosis characterizes a borderline condition of vitamin B_{12} deficiency. This would explain the presence of perosis in chicks fed high levels of soybean oil meal, as found by Carver et al. ('47) and Bird et al. ('46).

SUMMARY

A definite peak of embryonic mortality occurred at the 17th day of incubation in eggs from hens fed a diet deficient in vitamin B_{12}. The most characteristic symptom of deficiency present in the embryos was myoatrophy of the leg, a con-

dition which is characterized by atrophy of the muscles of the leg. The legs are slender, about normal in length and hemorrhagic in appearance.

A malposition, "head between the thighs," occurred with unusual frequency in eggs from hens fed a vitamin B_{12}-deficient diet.

Other anomalies associated with vitamin B_{12} deficiency in this study were hemorrhages of the embryo and allantois and perosis.

LITERATURE CITED

BIRD, H. R., MAX RUBIN, D. WHITSON AND S. K. HAYNES 1946 Effectiveness of dietary supplements in increasing hatchability of eggs and viability of progeny of hens fed a diet containing a high level of soybean oil meal. Poultry Sci., *25:* 285–293.

CARVER, J. S., JAMES MCGINNIS AND R. J. EVANS 1947 The occurrence of perosis in day-old chicks. Ibid., *26:* 91–93.

COUCH, J. R., W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 Relation of biotin to congenital deformities in the chick. Anat. Rec., *100:* 29–48.

CRAVENS, W. W. 1949 Fertility and Hatchability of Chicken and Turkey Eggs. Edited by L. W. Taylor. Chap. I. The nutrition of the breeding flock. John Wiley and Sons, Inc., New York, N. Y.

CRAVENS, W. W., W. H. MCGIBBON AND E. E. SEBESTA 1944 Effect of biotin deficiency on embryonic development in the domestic fowl. Anat. Rec., *90:* 55–64.

DANIEL, LOUISE J., FLORENCE A. FARMER AND L. C. NORRIS 1946 Folic acid and perosis. J. Biol. Chem., *163:* 349–350.

LANDAUER, W. 1948 The hatchability of chicken eggs as influenced by environment and heredity. Storrs Agr. Expt. Sta. Bull. 262.

OLCESE, ORLANDO, AND J. R. COUCH 1949a Unpublished data.

OLCESE, ORLANDO, J. R. COUCH AND CARL M. LYMAN 1949b The relation of unidentified factors to the nutrition of the mature domestic fowl. Poultry Sci.., *28:* 778.

———— 1950 Vitamin B_{12} concentrates in the nutrition of the mature domestic fowl. J. Nutrition, *41:* 73–87.

RICHARDSON, L. R., A. G. HOGAN AND O. N. MILLER 1942 The relation of biotin to perosis in chicks. Missouri Agr. Expt. Sta., Res. Bull. 343, pp. 3–10.

SKEGGS, H. R., J. W. HUFF, L. D. WRIGHT AND D. K. BOSSHARDT 1948 The use of *Lactobacillus leichmannii* in the microbiological assay of the "Animal Protein Factor." J. Biol. Chem., *176:* 1459–1460.

THE EFFECT OF PHYTATE AND OTHER FOOD FACTORS ON IRON ABSORPTION[1]

LEON M. SHARPE,[2] WENDELL C. PEACOCK,[3] RICHARD COOKE AND ROBERT S. HARRIS

WITH THE TECHNICAL ASSISTANCE OF HELEN LOCKHART, HELEN YEE AND GERTRUDE NIGHTINGALE

Departments of Biology, Food Technology and Physics of the Massachusetts Institute of Technology, Cambridge, and the Fernald State School, Waverly, Massachusetts

TWO FIGURES

(Received for publication November 22, 1949)

It has been postulated that phytates interfere with iron absorption by reacting with the iron as it passes through the intestinal tract, and that the insoluble iron phytate which is formed is unabsorbable (Widdowson and McCance, '42; McCance, Edgecombe and Widdowson, '43). Rolled oats, in common with other cereals, contain significant amounts of phytates. The present investigation is concerned with the effect of phytate — as contained, (a) in rolled oats, (b) in a typical breakfast which includes rolled oats, and (c) in sodium phytate — upon the absorption of radioactive iron in a group of adolescent boys. The results indicate that the phytate in rolled oats is relatively unavailable for the precipitation of food iron, and that the solids content of the food may have an important effect upon the absorption of iron from the intestinal tract.

[1] This research was supported by a grant from the Quaker Oats Company.

[2] Present address: Brookhaven National Laboratory, Upton, N. Y.

[3] Present address: Sloan-Kettering Institute, New York, N. Y.

EXPERIMENTAL

The radioactive isotopes used as tracers in the following experiments were either Fe^{55}, half-life 4 years, or Fe^{59}, half-life 44 days. Their preparation, either separately (by deuteron bombardment of Mn and Co targets, respectively) or as a mixture (by bombardment of iron phosphide), as well as their purification, has been described elsewhere (Peacock et al., '46; Hahn et al., '39; Balfour et al., '42). Approximately 3% of the x-rays resulting from the disintegration

TABLE 1

Composition of the test meals (in grams)

TEST MEAL	I	II	III	IV	V	VI	VII
Milk	200	200	200	200		200	200
Water					200		
Cooked rolled oats	285			173		285	285
White bread	34					56	
Egg omelet	75					75	
Tomato juice	150					150	
Sodium phytate			0.2				
Total wet weight	744	200	200.2	373	200	766	485
Total dry weight (calculated)	119	26	26	52	0	136	69

of Fe^{55} and 25% of the beta rays from the decay of Fe^{59} were detected by the differential G.M. tubes employed (Peacock et al., '46).

Seventeen boys, resident in a mental institution and with I.Q.'s ranging between 65 and 70, were selected for this study. The ranges and means of the groups were as follows: age, 12–17, 14, years; height 51–68, 60, inches; weight 69–136, 98, pounds; erythrocyte count 2.75–5.20, 4.50, millions/mm^3; and hemoglobin level, 60–104%, 93% (Sahli). All subjects except number 16 were in good health as judged by routine physical examinations and differential counts. Subject 16 had a low erythrocyte count and a low hemoglobin value;

nevertheless, it was subsequently observed that his response to the iron feedings was of the same order of magnitude as that of the other participants who did not show this anemic condition. All subjects were maintained on a daily vitamin supplement,[4] starting 6 weeks before the initial experiment and continuing until the last test was completed.

A total of 7 test breakfasts were consumed, each being ingested between 7:00 and 8:00 A.M. No other food was

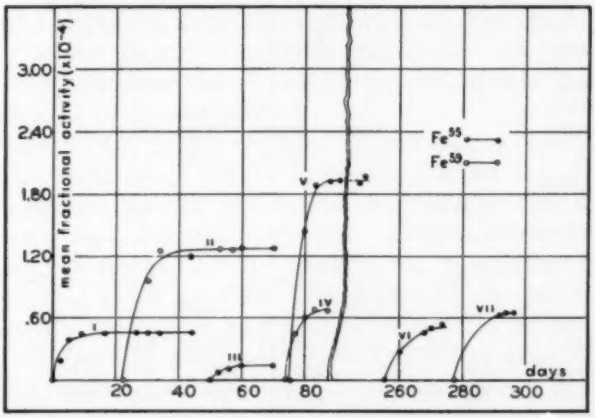


Fig. 1 Mean fractional activity as a function of time. Composition of test meals: I and VI, milk, rolled oats, tomato juice, egg omelet and white bread; II, milk; III, milk and sodium phytate; IV and VII, milk and rolled oats; and V, water.

eaten between 6:00 P.M. of the preceding evening and noon of the day when the test meal was given. The meals were fed in the order presented in table 1; the number of days elapsing between meals is indicated in figure 1. The components and portions of meal I (table 1) were selected as typical of an average breakfast eaten by adolescent boys in

[4] Supplement capsule contained: 5,000 U.S.P. vitamin A, 1,000 U.S.P. vitamin D, 3 mg thiamine, 0.3 mg riboflavin, 0.125 mg pyridoxine, 20 mg niacinamide, 3 mg calcium pantothenate, 5 mg vitamin E, and 50 mg ascorbic acid.

this country; this meal was repeated about 8 months later (meal VI). The remaining 5 meals (meals II, III, IV and VII) consisted of parts of these complete breakfasts, and the control meal (meal V) contained no food at all.

The iron content of all meals was approximately the same (table 2), being present either in the food components or as ferric chloride which was added to the milk or water and

TABLE 2

Iron, calcium and phytate content of test meals (in milligrams)

TEST MEAL	I	II	III	IV	V	VI	VII
Stable iron							
in ferric chloride		7.860	8.040	5.589	8.180		
in milk [1]		0.140	0.140	0.140			0.093
in complete meal	8.18					7.04	
in rolled oats				1.40			1.83
Radioactive iron [2]							
Fe^{55}	0.131		0.131		0.131	0.518	
Fe^{59}		0.311		1.098			5.708 [3]
Total iron	8.311	8.311	8.311	8.227	8.311	7.558	7.631
Total calcium	330	225	225	240	0.0	380	250
Phytic acid	203	0.0	203	86	0.0	203	203
Radioactivity (e.p.m. $\times 10^4$)	2.0	1.2	2.0	2.0	2.0	3.0	1.3

[1] Tables of Food Composition, U. S. Dept. of Agriculture, Misc. Publ. 572, 1945.
[2] Including stable carrier iron.
[3] Mixture of Fe^{59} and Fe^{55}; Fe^{59} alone measured.

distributed by stirring. The iron content of meals VI and VII was approximately 8% less than that of the other test meals.

In addition to the ferric chloride, an amount of an unstable iron isotope (table 2), either Fe^{59} or Fe^{55}, was added to the milk or water. In meal VII a mixture of the two isotopes was ingested but only Fe^{59} was measured. Either the chloride or citrate was employed. Though each subject participating

in a test meal ingested the same amount of radioactive material, the amount of activity ingested varied from 1.2×10^6 counts per minute in meal II to 3.0×10^6 counts per minute in meal VI. The reduction of the total iron, both stable and radioactive, was accomplished by adding an excess (32 mg) of ascorbic acid (Hahn et al., '45).

Meals I, VI and VII contained approximately the same quantities of phytate in the natural form in rolled oats, while meal IV contained approximately 40% of this amount (table 2). The phytate content of meal III was the same as that of meal I; however, it was present as sodium phytate, which is very soluble in water and fully "available" to react with iron. Meal II consisted of one food, milk; it was analyzed and found to be free of phytate.

Samples of the food were taken at random. Aliquots were ashed (Association of Official Agricultural Chemists, '45) and the residue analyzed for iron content by the bipyridine method of Koenig and Johnson ('42) and for calcium content by the A.O.A.C. method ('45). Phytate was estimated by first extracting it from the food with 10% sodium sulfate in a 1.2% hydrochloric acid solution (Rather, '17), precipitating it as iron phytate, and then measuring the residual iron in the supernatant (Earley, '44). The supernatant iron was reduced with sulphur dioxide according to the method of Koenig and Johnson ('42), rather than by the Walden reductor used by Earley.

Samples of blood were taken in heparin from the antecubital area of the subject at convenient intervals (see fig. 1) after each experimental meal. Each sample (10 ml) was centrifuged at 3,000 r.p.m. for 30 minutes and the hematocrits recorded. The plasma was removed by suction and generally discarded. In a few cases it was checked for activity, which, as expected, was absent.

Irrespective of which isotope was to be counted, enough inactive iron as ferric chloride was added to each red cell specimen prior to wet digestion to bring the total iron content to 10 mg (2.6 mg/cm^2). In making these additions it

was assumed that each milliliter of red cells contained 1 mg of iron. The samples were then wet ashed and electroplated onto copper planchets according to the procedure described by Peacock et al. ('46). The radiations from the mixed isotopes were selectively counted by utilizing the differential counters described in the same paper.

RESULTS

All radioactivity measurements were initially recorded in terms of "unit activity" or "U." This quantity, which is independent of decay, may be defined as:

$$U = \frac{\text{net counts/min./ml red cells}}{\text{net counts/min. fed}}.$$

The net counts per minute fed is the "standard." When activity was present in the cells as a result of previous experiments which utilized the same isotope, a correction was made. The corrected unit activity, designated the "fractional activity" and represented by "F," was calculated according to the following equation:

$$F = U_2 - U_1(S_1/S_2). \tag{1}$$

S_1 and S_2 are the standards from the two experimental meals. In order to obtain the correct fractional activity, S_1 and S_2 were measured at the same time. Thus, the activity for each isotope was expressed in terms of U and F, where $U = F$ if the blood was free of activity prior to the experiment.

While the rate of accumulation of iron in the red cells is of interest, only the maximum fractional activities can be used as an index of absorption. It has been reported that a maximum is attained in 8 days following ingestion (Moore et al., '44; Hahn et al., '45) and not until 21 days following parenteral administration (Gibson et al., '46b). In agreement with Moore and Hahn, a maximum was attained in the present investigation approximately 8 days following each meal (fig. 1). Consequently, only those radioactivity data obtained from a blood sample drawn a minimum of 8 days

following an experimental feeding have been used in our calculations.

Once iron is incorporated into the red cells it may be detected in the erythrocytes at a constant level for many months (Hahn, Bale and Balfour, '42) provided there has been no dilution of the radioactive red cells by non-radioactive erythrocytes. The data obtained from the last 4 blood samples following meal V are in agreement with this observation. These blood samplings were spread over 170 days, with 165 days intervening between the second and third. A comparison, subject by subject, of the mean U value of the first and second of these samples with that obtained from the 3rd and 4th showed a minimum and maximum ratio of 0.91 and 1.13, with a mean of 1.00, for 12 of the 17 subjects. The remaining 5 subjects were not included in this comparison because less than 4 blood samples were obtained from them. All maximum U values for each subject, resulting from this meal, were therefore comparable irrespective of elapsed time, and were averaged in calculating the fractional activity. Blood activity data collected following each of the other meals were distributed over a maximum of only 36 days (meal II), so that these data were averaged for each meal.

To illustrate one method used in analyzing the data, the results of meals II (milk only) and V (control) are treated here in detail. Because of the wide variation in the individual response to the same dose of iron, and, further, because the standard deviation is a function of that response, it was necessary to normalize the maximum U data resulting from the two meals for the purpose of calculating the standard deviation, σ. The latter was found to be $\pm$ 5.4% and $\pm$ 8.7% for a single maximum unit activity in meals II and V, respectively. When the maximum unit activities for a subject were averaged, $\sigma U = \sigma/\sqrt{n}$, the σU for meals II and V did not exceed $\pm$ 3.8 and $\pm$ 3.9%, respectively.

Having established the significance (σU) of each average maximum U for each subject following both meals, the corresponding average maximum F data and their significance

were calculated (table 3). In the case of the milk meal (II), no Fe^{59} had been fed in prior experiments so that $U \pm \sigma U = F \pm \sigma F$. Since the subjects had already taken part in other Fe^{55} experiments, it was necessary to derive F for the water meal (V) by means of equation 1. For this calculation it was found that the constant S_1/S_2 was 0.352; also

$$\sigma F = \sqrt{(\sigma U_2)^2 + (\sigma U_1 \times S_1/S_2)^2}.$$

Inasmuch as the blood volumes were not considered to have changed appreciably over the experimental period, the average F for each subject resulting from each of the two experimental conditions was used as a basis of comparison. Thus, each participant acted as his own control. Inherent in such a comparison is the assumption that different radio-

TABLE 3

Comparison by subjects of fractional activities ($\times 10^{-4}$) resulting from water and milk test meals

SUBJECT NO.	WATER MEAL [1]		MILK MEAL [2]		$\Delta F/\sigma\Delta F$
	Ave. F_w	σF_w	Ave. F_m	σF_m	
1	0.72	0.072	1.33	0.047	— 7.1
2	2.12	0.070	1.44	0.051	+ 7.8
3	1.23	0.044	0.861	0.030	+ 7.0
4	3.06	0.100	1.41	0.050	+ 14.7
5	1.64	0.046	1.10	0.039	+ 9.0
6	2.37	0.074	1.01	0.036	+ 16.6
7	1.74	0.105	1.61	0.057	+ 1.1
8	2.12	0.079	1.41	0.050	+ 7.7
9	2.59	0.081	1.10	0.039	+ 16.7
10	3.36	0.105	1.84	0.065	+ 12.4
11	0.459	0.040	1.16	0.041	— 14.6
12	2.43	0.106	1.25	0.044	+ 10.3
13	1.80	0.086	1.73	0.061	+ 0.7
14	1.96	0.113	0.396	0.015	+ 13.7
15	1.38	0.040	0.904	0.035	+ 9.1
16	1.64	0.057	0.933	0.033	+ 10.6
17	2.33	0.084	1.88	0.066	+ 4.2

[1] Data from subjects 1, 7, 12, 13, 14 represent an average from two blood specimens each; the remaining averages were obtained from 4 blood specimens.

[2] Data from subjects 14 and 15 represent an average of 5 blood specimens each; the remaining averages were obtained from 6 blood specimens.

active isotopes of iron, Fe^{55} and Fe^{59}, are metabolized by the body in a non-discriminatory manner.

To determine whether or not the difference between the two F values in a subject was due to chance, let F_w and F_m refer to the water (V) and milk (II) meals, respectively. Then $F_w - F_m = \Delta F$ and

$$\sigma\Delta F = \sqrt{(\sigma F_w)^2 + (\sigma F_m)^2}.$$

If $\Delta F/\sigma\Delta F > 3$, then the difference may be considered as not due to chance. Table 3 lists the data for the 17 subjects. It is evident that in 12% of the cases (subjects 7 and 13) the

TABLE 4

Percentage of radioactive iron absorbed from experimental meals

MEAL NO.	FOOD	WT. OF MEAL		TOTAL SUBJECTS	AVE. FRACTIONAL ACTIVITY	IRON ABSORBED
		Wet	Dry			
		gm	*gm*		$\times 10^{-4}$	%
V	Water	200	0	10	1.90	26.9
II	Milk	200	26	10	1.31	17.4
IV	Milk and oatmeal	373	52	10	0.763	9.81
VII	Milk and oatmeal	485	69	10	0.643	8.84
VI	Complete meal	766	136	10	0.412	5.76
I	Complete meal	744	119	10	0.402	4.43
III	Milk and Na phytate	200	26	10	0.127	1.74

iron was absorbed equally well from both meals; in another 12% (subjects 1 and 11) the absorption was better from meal II than from meal V; and in 76% of the cases the presence of milk reduced the absorption of iron significantly.

The results of a group of meals can be interpreted more readily if the average fraction of the radioactive iron utilized for hemoglobin is used as an index of absorption. This has been done for each meal by making use of the following relationship:

(2) $$\text{Average per cent absorbed} = 80 \times W \times H \times F \times 0.85 \times 100.$$

In this equation it is assumed that an individual contains 80 ml of whole blood per kilogram of body weight (Hahn

et al., '45). H represents the hematocrit, F the fractional activity and 0.85 a correction factor (Gibson et al., '46a). In making our calculations we used the average body weight of the group of subjects under consideration, their average hematocrit, and their average fractional activity. It is to be noted that the last two meals contained about 8% less iron than the preceding 5 meals (table 2) and that all 17 subjects did not participate in each of the 7 meals. We are of the opinion that the difference in iron content is too small to be significant. In view of the wide difference in individual response to equal doses of iron, a factor that has been emphasized by some investigators (Hahn et al., '45), we calculated the average iron utilized from each meal on the basis of those data obtained from the 10 subjects who took part in all 7 meals (table 4).

Various fractions of the maximum inhibition in iron absorption were assigned. Since the results obtained from feeding the two complete breakfasts (I and VI) agreed so well, the absorptions were averaged in making this calculation. Thus, the 5 food components combined reduced iron absorption by four-fifths. Milk alone reduced it by one-third, milk and rolled oats by two-thirds and sodium phytate by fourteen-fifteenths.

DISCUSSION

Data derived from the maximum fractional activity have been used to indicate the degree of absorption (Hahn, Bale, Lawrence and Whipple, '39; Hahn et al., '43; Moore et al., '44; Hahn et al., '45; Darby et al., '47). Quantities so calculated represent only that iron incorporated in the hemoglobin, and the portion of the absorbed iron finding its way into other tissues is neglected. Dubach and her associates ('48) have found this amount to be small in the case of normal humans and have characterized the iron utilized for hemoglobin formation as the best measure of absorbed iron available. Consequently, the fractional activity and the quantities based upon it are regarded by us as an acceptable index of absorption when applied to normal humans.

The absorption of iron was greatest from the distilled water (meal V), presumably because it contained no interfering substances. Milk reduced iron absorption appreciably; in fact, about as much as the rolled oats, which contained phytate. A series of analyses of milk failed to demonstrate the presence of phytate. The reduction in the absorption of iron caused by milk cannot be due to a difference in availability, for all of the iron in both the milk (II) and control (V)

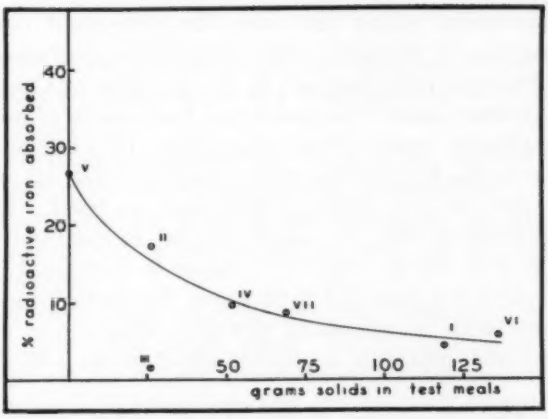


Fig. 2 Relationship between the solids content of the test meals and the absorption of radioactive iron. Composition of test meals: I and VI, milk, rolled oats, tomato juice, egg omelet and white bread; II, milk; III, milk and sodium phytate; IV and VII, milk and rolled oats; and V, water.

meals had been added as ferric chloride. Since rolled oats and milk reduced absorption only twice as much as milk alone, it is possible that, under the conditions of the experiment, phytate was not an important factor in reducing the absorption of iron. Further support for this conclusion is to be found in the fact that a 40% reduction in the amount of rolled oats did not materially affect iron absorption.

Rolled oats are almost always eaten with milk and the latter is a particularly rich source of calcium. In fact, meals I, III, IV, VI and VII contained 5.3, 3.7, 9.2, 5.7 and 3.5

times, respectively, as much calcium as is required to precipitate all of the phytate they contained as pentacalcium phytate (Hoff-Jørgensen, '44). It is suggested that, in view of the apparent lack of interference with iron absorption by the phytate occurring naturally in oats, the calcium in the milk preferentially combined with phytate, making it unavailable for iron precipitation. It would appear, however, that such was not the case when sodium phytate was added in the pure state (meal III).

In this study rolled oats was the only food component which contained phytates. The progressive decrease in iron absorption with each increase in the quantity of the different foods ingested makes it appear that there were other factors which militated against iron absorption. It is not possible to conclude on the basis of the study whether these other factors include dry weight, total protein, and so forth. However, forceful evidence that the solids content of the food ingested (table 4) may be an important factor is contained in figure 2. The point for meal III falls below the plotted line and, in agreement with the results of McCance, Edgecombe and Widdowson ('43), indicates a positive effect of the added sodium phytate.

Since the absorption of iron from water (meal V) was approximately 5 times greater than that from the breakfasts (meals I and VI), it is apparent that medicinal iron administered in the morning before any food is eaten would be much more effective than iron taken with meals.

SUMMARY

1. The effect of 7 test meals upon the absorption of either reduced Fe^{55} or reduced Fe^{59} fed to adolescent boys was measured.

2. The test meals consisted of (I, VI) milk, rolled oats, tomato juice, white bread and omelet; (II) milk; (III) milk and sodium phytate; (IV, VII) milk and rolled oats; and (V) water. Each meal contained approximately the same amount of iron. Meals I, VI and VII contained the same level

of natural phytate in rolled oats; meal III contained the same quantity of phytate, as the sodium salt; meal IV contained approximately half as much rolled oats and phytate as meal VII; phytate was absent from meals II and V.

3. The following percentages of radioactive iron were absorbed from these meals: (I) 4.43, (II) 17.4, (III) 1.74, (IV) 9.81, (V) 26.9, (VI) 5.76 and (VII) 8.84.

4. Milk reduced the absorption of iron by one-third. This was not due to the action of phytates, for the milk contained none.

5. Rolled oats and milk reduced the absorption of iron by two-thirds. Thus, rolled oats interfered no more than did milk.

6. There was no correlation between the phytate content of rolled oats and the reduction in iron absorption.

7. Sodium phytate reduced the absorption of iron by 15-fold, indicating that added soluble phytates can interfere with iron absorption.

8. The absorption of iron from the breakfast was one-fifth that from the water meal. Medicinal iron should be more effective if administered between meals.

9. Evidence is presented for the inverse correlation of iron absorption with the solids content of the test meals.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official and Tentative Methods of Analysis.

BALFOUR, W. M., P. F. HAHN, W. F. BALE, W. T. POMMERENKE AND G. H. WHIPPLE 1942 Radioactive iron absorption in clinical conditions: normal, pregnancy, anemia and hemochromatosis. J. Exp. Med., *76:* 15.

DARBY, W. J., P. F. HAHN, M. M. KASER, R. C. STEINKAMP, R. M. DENSEN AND M. B. COOK 1947 The absorption of radioactive iron by children 7–10 years of age. J. Nutrition, *33:* 107.

DUBACH, R., S. T. E. CALLENDER AND C. V. MOORE 1948 Studies in iron transportation and metabolism. VI. Absorption of radioactive iron in patients with fever and with anemias of varied etiology. Blood, *3:* 526.

EARLEY, E. B. 1944 Determining phytin phosphorus. Ind. Eng. Chem., *16:* 389.

GIBSON, J. G., W. C. PEACOCK, A. M. SELIGMAN AND T. SACK 1946a Circulating red cell volume measured simultaneously by the radioactive iron and dye methods J. Clin. Invest., *25:* 838.

GIBSON, J. G., S. WEISS, R. D. EVANS, W. C. PEACOCK, J. W. IRVINE, JR., W. M. GOOD AND A. F. KIP 1946b The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. Ibid., *25:* 616.

HAHN, P. F., W. F. BALE AND W. M. BALFOUR 1942 Radioactive iron used to study red cells over long periods. Am. J. Physiol., *135:* 600.

HAHN, P. F., W. F. BALE, E. O. LAWRENCE AND G. H. WHIPPLE 1939 Radioactive iron and its metabolism in anemia. J. Exp. Med., *69:* 739.

HAHN, P. F., W. F. BALE, J. F. ROSS, W. M. BALFOUR AND G. H. WHIPPLE 1943 Radioactive iron absorption by the gastrointestinal tract; influence of anemia, anoxia, and antecedent feeding; distribution in growing dogs. Ibid., *78:* 169.

HAHN, P. F., E. JONES, R. C. LOWE, G. E. MENEELY AND W. C. PEACOCK 1945 The relative absorption and utilization of ferrous and ferric iron in anemia as determined with the radioactive isotope. Am. J. Physiol., *143:* 191.

HOFF-JØRGENSEN, E. 1944 Investigations on the solubility of calcium phytate. Kgl. Danske Videnskab. Selskab, Math.-fys Medd., *21:* 7. (Through Chem. Abstr., 1946.)

KOENIG, R. A., AND C. R. JOHNSON 1942 Spectrophotometric determination of iron. II. The use of 2-2' bipyridine. J. Biol. Chem., *143:* 159.

MCCANCE, R. A., C. N. EDGECOMBE AND E. M. WIDDOWSON 1943 Phytic acid and iron absorption. Lancet, *2:* 126.

MOORE, C. V., R. DUBACH, V. MINNICH AND H. K. ROBERTS 1944 Absorption of ferrous and ferric radioactive iron by human subjects and dogs. J. Clin. Invest., *23:* 755.

PEACOCK, W. C., R. D. EVANS, J. W. IRVINE, JR., W. M. GOOD, A. F. KIP, S. WEISS AND J. G. GIBSON, JR. 1946 The use of two radioactive isotopes of iron in tracer studies of erythrocytes. Ibid., *25:* 605.

RATHER, J. B. 1917 Determination of phytin phosphorus in plant products. J. Am. Chem. Soc., *39:* 2506.

WIDDOWSON, E. M., AND R. A. MCCANCE 1942 Iron exchanges of adults on white and brown bread diets. Lancet, *1:* 588.

OBSERVATIONS ON NIACIN, RIBOFLAVIN, ALLANTOIN, ASCORBIC ACID, AND VITAMIN A DURING ANABOLISM INDUCED BY HORMONES [1]

WILLIAM T. BEHER AND O. H. GAEBLER

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, and the Department of Chemistry, Wayne University, Detroit, Michigan

(Received for publication February 7, 1950)

The apparent concentration of some tissue enzymes is altered by secreted or administered hormones. Effects of thyroid (Klein, '39), pituitary (Fraenkel-Conrat et al., '43; Li et al., '47) and steroid hormones (Kochakian et al., '48) have been reported. In this laboratory various changes in tissue enzyme concentrations which occur when growth is arrested by hypophysectomy or stimulated by injection of anterior pituitary growth preparations have recently been investigated (Mathies et al., '49b; Bartlett and Gaebler, '49a, b; Gaebler et al., '49). The close relationship between vitamins and enzyme systems suggested the present study, in which indirect evidence for the synthesis of coenzymes during induced gain in weight is considered.

If synthesis of coenzymes I and II, or flavin nucleotides, is accelerated by testosterone propionate or by anterior pituitary growth preparations, one might expect diminished excretion of N^1-methylnicotinamide and riboflavin. Although adenine is also present in the coenzymes mentioned, decreased output of its end-product, allantoin, would hardly be anticipated. The total amount of free and combined purines which

[1] The data in this paper are taken from a thesis submitted by one of us (W. T. B.) to the Graduate Council of Wayne University in partial fulfillment of requirements for the degree of Doctor of Philosophy in Chemistry.

yield allantoin is large, and the animal can synthesize the adenine required for production of coenzymes and nucleoproteins. With these considerations in mind, we determined the output by dogs of N^1-methylnicotinamide, riboflavin, and allantoin in urine, and the concentration of coenzymes I and II in erythrocytes, during induced anabolism. This phase of our investigation also extended an earlier study on the effect of growth preparations on the excretion of nitrogenous substances (Gaebler, '33).

Since there were definite indications that testosterone propionate affects the metabolism of niacin and riboflavin, we also performed a number of experiments to determine its effect on the excretion and blood concentration of ascorbic acid and the plasma level of vitamin A. The results with ascorbic acid, which the dog can synthesize and which appears to be required in the free form rather than as a coenzyme component, were of particular value in determining the significance of the positive findings in the case of niacin, riboflavin, and vitamin A.

EXPERIMENTAL

Five adult bitches, well trained in our routine during long preliminary periods, were used in our experiments. Dietary requirements were determined for each animal. The metabolism cages were equipped with polished stainless steel drain pans having a steep slope to the center drain, and collection periods were terminated by catheterizing and washing the bladder. Thereafter, the animals were allowed about 30 minutes of exercise.

Total nitrogen was determined by a modification of the Kjeldahl method. Samples contained about 5 mg of nitrogen, and 0.02 N acid and alkali were used for titration. Urea and ammonia nitrogen were determined by the method of Van Slyke and Cullen ('14). Fluorometric methods utilizing a model 12B Coleman photofluorometer were used for determining N^1-methylnicotinamide (Huff and Perlzweig, '47), coenzymes I and II (Levitas et al., '47), and riboflavin (Najjar, '41). Allantoin was determined by the method of Young

and Conway ('42), and ascorbic acid by the method of Roe and Kuether ('43).

Diet 6 consisted of casein, 36%; cracker meal, 36%; corn oil, 20%; powdered brewers' yeast, 4%; and Phillips-Hart ('35) salt mixture, 4%. The modified Goldberger diet was that of Koehn and Elvehjem ('37) altered further by substituting corn oil for cottonseed oil and omitting cod liver oil. Except where other amounts are stated, all animals received three drops of haliver oil daily.[2]

RESULTS

That anabolism was induced in all experiments was indicated by gain in weight, diminished output of nitrogen with the intake constant, and an increase in water intake greater than the rise in urine volume. Data on weight and nitrogen output are given in all tables. Water intake and urine volumes were recorded daily, and average values are given in the footnotes of several tables.

Niacin

Five experiments, each performed on a different animal, yielded entirely concordant results. Three of these are presented in tables 1, 2, and 3. Injection of an anterior pituitary growth preparation (table 1) produced the usual fall in nitrogen output, due to decreased excretion of urea and accompanied by some increase in urinary ammonia (Gaebler, '33; Bartlett and Gaebler, '49a). Excretion of N^1-methylnicotinamide fell markedly and subsequently, during days 18 to 20, rose far above the initial control level. When anabolism was induced with testosterone propionate, whether under conditions of adequate niacin intake (table 2) or moderate niacin deficiency (table 3), a fall in N^1-methylnicotinamide output was also observed. In neither of these experiments (tables

[2] The anterior pituitary growth preparation was supplied by Parke, Davis and Company. Testosterone propionate was purchased from Rare Chemicals in a sesame oil solution containing 25 mg per milliliter.

TABLE 1

Effect of anterior pituitary growth preparation on urinary total nitrogen, urea, ammonia, and N¹-methylnicotinamide

DAYS	WEIGHT	TOTAL N	UREA N	AMMONIA N	N¹-METHYL-NICOTINAMIDE
	kg	*gm/day*	*gm/day*	*gm/day*	*mg/day*
1–5	19.18	10.43	8.57	0.59	11.3
6–8	19.26	10.37	8.51	0.62	10.5
9–11	19.11	10.57	8.57	0.56	10.8
12 [1]	19.52	10.43	8.42	0.42	4.3
13	19.41	8.74	7.60	0.72	2.2
14	19.30	8.80	7.95	0.70	2.4
15	19.41	9.75	7.95	0.67	2.1
16	19.30	10.30	8.77	0.73	3.5
17	19.30	11.20	9.00	0.70	12.2
18–20	19.10	12.07	9.56	0.62	18.1
21–23	18.99	12.70	10.37	0.69	17.0

[1] Two hundred milligrams of anterior pituitary growth preparation Rx 099858 suspended in 0.9% saline were injected subcutaneously at 10 A.M.

Dog 45, used in this experiment, was a German shepherd, receiving 200 gm of diet 6 daily.

During the control periods preceding the injection, the average daily water intake was 672 ml and the average daily urine volume, 389 ml. These averages rose to 1,088 ml and 628 ml, respectively, during the 6 days following the injection.

TABLE 2

N¹-methylnicotinamide output and blood concentration of coenzymes I and II after testosterone propionate, with niacin intake adequate

DAYS	WEIGHT	TOTAL N	N¹-METHYL-NICOTINAMIDE	CELL VOLUME	COENZYMES I AND II
	kg	*gm/day*	*mg/day*	%	*μg/ml cells*
1–5	13.53	10.02	11.8	48	69
6–10	13.62	9.96	12.3	48	66
11–15	13.79	9.76	11.3	51	74
16 [1]	13.82	9.77	8.7		
17 [1]	13.93	8.69	5.1	48	68
18 [1]	14.16	8.40	5.3	47	75
19 [1]	14.16	8.60	3.7		
20	14.16	8.40	4.3	47	60
21	14.04	8.46	5.2		
22–24	14.16	9.68	6.2	47	67
25–27	14.27	9.67	9.7	50	73

[1] Fifty milligrams of testosterone propionate were injected daily, intramuscularly. Dog 49 used in this experiment was a hound receiving 200 gm of diet 6 daily.

2 and 3) did the concentration of coenzymes I and II in red blood cells rise. Assuming that diminished excretion of the niacin derivative is due to synthesis of coenzyme I, one may calculate the amount of the latter compound expected. In the experiment shown in table 2, excretion of N^1-methylnicotinamide during days 16 to 27 fell short of the amount predictable from the initial control period by a total of 61.6 mg.

TABLE 3

Output of N^1-methylnicotinamide and blood concentration of coenzymes I and II after injecting testosterone propionate during moderate niacin depletion

DAYS	WEIGHT	TOTAL N	N^1-METHYL-NICOTINAMIDE	CELL VOLUME	COENZYMES I AND II
	kg	*gm/day*	*mg/day*	*%*	*μg/ml cells*
1–6	10.45	6.06	4.5	45	68
7–12	10.38	6.13	4.6	47	67
13–17	10.45	6.01	3.6	45	61
18 [1]	10.45	6.13	2.7		
19 [1]	10.45	5.62	2.9		
20 [1]	10.45	5.39	2.4	49	59
21 [1]	10.45	5.62	1.9		
22	10.45	4.65	1.6	45	71
23	10.45	5.01	1.0		
24–29	10.45	5.90	1.3	46	70
30–36	10.45	7.70	5.1		

[1] Fifty milligrams of testosterone propionate were injected daily, intramuscularly.

Dog 40 used in this experiment was a bull terrier receiving 200 gm of modified Goldberger diet daily.

During days one to 17, the average daily water intake was 852 ml, the average daily urine volume, 575 ml; the corresponding averages for days 18 to 23 were 1,012 and 625 ml.

This would be equivalent to 298 mg of coenzyme I, or 21 μg per gram of animal — an amount that should be readily detectable. These findings are considered further in the discussion section which follows.

A point which is apparent in our records of daily analyses, but not in the condensed tables, is that diminished output of N^1-methylnicotinamide after testosterone propionate injections persists longest when the control level of excretion is low. In 4 experiments, including two shown in tables 2

and 3, the average excretion of the niacin derivative before injections was 11.8, 11.5, 6.7, and 4.2 mg per day. In the first two experiments excretion remained below the control level for an average of 9 days; in the last two, for 16 days.

TABLE 4

Effect of testosterone propionate on excretion of riboflavin and allantoin

DOG NO.	DAYS	WEIGHT	TOTAL N	RIBOFLAVIN	ALLANTOIN
		kg	*gm/day*	*μg/day*	*mg/day*
40	10–12	12.62	9.86	213	430
	13–15	12.79	10.13	187	430
	16–18 [1]	12.83	9.78	176	435
	19–21 [1]	13.17	9.32	105	474
	22–24	13.00	10.54	117	444
	25–27	12.90	10.90	327	426
	28–30	12.95	11.30	565	453
	31–33	12.86	10.17	293	428
48	7–9	16.45	8.40	81	421
	10–12	16.53	8.67	81	429
	13–15	16.60	8.77	78	435
	16–18 [1]	16.68	8.30	78	448
	19–21 [1]	17.05	6.97	67	445
	22–24	17.01	7.66	60	431
	25–27	16.86	8.95	76	489
	28–30	16.93	9.25	83	452
	31–33	16.86	8.81	78	468

[1] Each animal received 50 mg of testosterone propionate daily, intramuscularly, on days 16 to 19 inclusive.

Dog 40, used in the first of the above experiments, was a bull terrier receiving 200 gm of diet 6 daily; dog 48 was a collie receiving 175 gm of diet 6 daily.

Riboflavin

In both experiments shown in table 4, excretion of riboflavin decreased during the period of anabolism induced by testosterone propionate (days 16 to 24) and subsequently (days 28 to 30) rose above the control level. These changes are more striking in the case of dog 40, which excreted much more riboflavin than did dog 48. The animals received the same diet and the difference in food intake, recorded in the footnote of table 4, was not large.

Allantoin

No consistent change in the output of this compound occurred during the two experiments with testosterone propionate recorded in table 4. In the experiment on dog 40 the average for the three-day period beginning on the 19th day

TABLE 5

Effect of testosterone propionate on urinary excretion and blood concentration of ascorbic acid

DOG NO.	DAYS	WEIGHT	TOTAL N	ASCORBIC ACID	
				Urine	Blood
		kg	*gm/day*	*mg/day*	*mg/100 ml*
40	1–3	10.94	10.50	42	0.68
	4–6	11.10	10.50	36	0.62
	7–10	11.19	10.26	42	0.61
	11–14 [1]	11.39	9.27	35	0.62
	15–17	11.75	9.10	33	0.68
	18–20	11.89	10.80	38	0.64
	21–23	11.91	11.00	44	0.56
48	6–8	15.62	8.87	63	0.60
	9–11	15.62	8.80	78	0.60
	12–16	15.62	9.42	72	0.59
	17–19 [1]	15.78	8.58	63	0.62
	20–22	16.04	7.33	62	0.61
	23–26	16.05	8.19	80	0.59
	27–32	15.99	8.81	82	0.59

[1] Each dog received 50 mg of testosterone propionate daily, intramuscularly, for 4 days beginning on the 11th day in the first, and on the 17th day in the second, experiment.

For diets, see footnote to table 4.

The experiment on dog 40 shown in this table followed the one presented in table 3 and preceded on the one in table 4. Dog 40 gained weight during this period.

was somewhat high, while in the case of dog 48 a similar average value was observed during the three-day period beginning on the 25th day.

Ascorbic acid

The average daily excretion of ascorbic acid, in two experiments shown in table 5, reached its lowest point during

the period when the decrease in total nitrogen output following injection of testosterone propionate was greatest. It is, however, evident at a glance that changes in the excretion of ascorbic acid are less decisive than those of N^1-methylnicotinamide and riboflavin. Blood ascorbic acid was unchanged.

Vitamin A

In an experiment in which the effects on weight and nitrogen retention were the same as in those presented in the tables, the vitamin A concentration in the plasma during the 7 days preceding administration of testosterone propionate averaged 195 mg %. During 4 days of hormone injection, and one additional day, the average value was 158 mg %; thereafter, it returned to the control level. The very high vitamin A concentration seen in the plasma of dogs receiving three drops of haliver oil daily led us to reduce the amount to two drops in this experiment, and it is evident that a further reduction could be made.

Statistical significance of results

Since condensed tables have been presented, the results of statistical analysis of the original data (Beher, '50), in which results of daily analysis are given, appear of interest. In the 5 experiments on 5 different dogs in which N^1-methylnicotinamide was determined, the probability that the diminished output during induced growth was due to chance was between 10^{-2} and 10^{-3} in the experiment shown in table 3, and below 10^{-9} in the other 4. In the two experiments with riboflavin, this probability was below 10^{-3} in both experiments; in the case of ascorbic acid, it was between 10^{-2} and 10^{-3} in the first experiment of table 5 and 0.28 in the second experiment (Fisher, '48).

DISCUSSION

Diminished catabolism of amino acids, indicated by the fall in urea excretion, is the principal change to be deduced from

analysis of urine during induced anabolism. It is, however, evident that minor nitrogenous constituents, such as N^1-methylnicotinamide and riboflavin, also fall. While this finding may be due to diminished absorption of niacin and riboflavin, it seems more logical to ascribe it to synthesis of coenzymes I and II and flavin nucleotides. The coenzyme concentration of red cells, unaltered in our experiments, is also unchanged in niacin depletion, while that of liver and muscle is lowered (Axelrod et al., '39). Investigation of the effects of anabolic hormones on the coenzyme content of liver and muscle would seem to be indicated, and is contemplated. The fall in ascorbic acid output, though less definite than that of N^1-methylnicotinamide and riboflavin, clearly indicates that while diminished excretion of the latter substances suggests synthesis of coenzymes, this evidence alone is not sufficient. Diminished output does provide some evidence that the increased vitamin requirement of growing animals, which is generally recognized, also applies during induced growth.

A related phenomenon is diminished excretion of creatine, of endogenous origin and therefore not subject to changes in absorption, when rapid growth is induced by increasing protein intake after a period of protein restriction (Harding and Gaebler, '23). Fall in the excretion of creatine from a dietary source is similarly seen in some experiments with anterior pituitary growth preparations (Gaebler and Bartlett, '39). Increased creatine excretion after methyl testosterone, which also has anabolic effects, is a notable exception, but creatine, like the purine precursors of allantoin and like ascorbic acid in the case of the dog, is synthesized in the body. Changes in excretion thus amount to an algebraic sum of changes in utilization and synthesis.

Allantoin, in the present study, behaved like creatinine (Gaebler, '33) in that it was unaffected by a brief period of anabolism.

SUMMARY

1. In adult bitches the excretion of N^1-methylnicotinamide decreases markedly during periods of anabolism induced by anterior pituitary growth preparations or testosterone propionate.

2. Coenzymes I and II in the red blood cells do not increase during this apparent storage of niacin.

3. Riboflavin output also decreases following administration of testosterone propionate.

4. Decreases in the excretion of ascorbic acid during similar experiments were significant but less striking; blood ascorbic acid was unchanged.

5. In a single experiment, the concentration of vitamin A in blood fell during the period when testosterone propionate produced its effect.

6. Allantoin excretion remained unchanged in these experiments.

7. The increase in vitamin requirements during normal growth may also be demonstrable during induced growth.

LITERATURE CITED

AXELROD, A. E., R. J. MADDEN AND C. A. ELVEHJEM 1939 The effect of a nicotinic acid deficiency upon the coenzyme I content of animal tissues. J. Biol. Chem., *131:* 85.

BARTLETT, P. D., AND O. H. GAEBLER 1949a Studies on the mechanism of nitrogen storage. II. Effects of anterior pituitary growth hormone preparations on kidney glutaminase. Ibid., *181:* 523.

———— 1949b Studies on the mechanism of nitrogen storage. III. The effects of anterior pituitary growth hormone preparations on the pyruvate activated deamidation of glutamine in liver tissue. Ibid., *181:* 529.

BEHER, W. T. 1950 Analytical and metabolic studies concerning steroid hormones. Thesis, Wayne University, Detroit, Mich.

FISHER, R. A. 1948 Statistical Methods for Research Workers. Oliver and Boyd, Edinburgh, Scotland, 10th ed.

FRAENKEL-CONRAT, H., M. E. SIMPSON AND H. M. EVANS 1943 Effect of hypophysectomy and of purified pituitary hormones on the liver arginase activity of rats. Am. J. Physiol., *138:* 439.

GAEBLER, O. H. 1933 Some effects of anterior pituitary extracts on nitrogen metabolism, water balance, and energy metabolism. J. Exp. Med., *57:* 349.

GAEBLER, O. H., AND P. BARTLETT 1939 Effects of anterior pituitary preparations and iodine on nitrogen excretion, creatinuria, and basal metabolism. J. Biol. Chem., *129:* 559.

GAEBLER, O. H., J. C. MATHIES AND L. PALM 1949 The effect of growth hormone upon liver and kidney D-amino acid oxidase and upon muscle succinic acid dehydrogenase. Endocrinology, *45:* 267.

HARDING, V. J., AND O. H. GAEBLER 1923 The influence of the positive nitrogen balance upon creatinuria during growth. J. Biol. Chem., *57:* 25.

HUFF, J. W., AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. Ibid., *167:* 157.

KLEIN, J. R. 1939 Effect of thyroid feeding and thyroidectomy on the oxidation of amino acids by rat kidney and liver. Ibid., *128:* 659.

KOCHAKIAN, C. D., E. E. GARBER AND M. N. BARTLETT 1948 Effect of estrogen alone and in combination with testosterone on the body and organ weights and the arginase and phosphatases of the organs of the mouse. Am. J. Physiol., *155:* 265.

KOEHN, C. J., JR., AND C. A. ELVEHJEM 1937 Further studies on the concentration of the antipellagra factor. J. Biol. Chem., *118:* 693.

LEVITAS, N., J. ROBINSON, F. ROSEN, J. W. HUFF AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. III. A rapid fluorometric method for the determination of the total pyridine nucleotides in the red blood cells. Ibid., *167:* 169.

LI, C. H., C. KALMAN AND H. M. EVANS 1947 The effect of the hypophyseal growth hormone on the alkaline phosphatase of rat plasma. Ibid., *169:* 625.

MATHIES, J. C., AND O. H. GAEBLER 1949a The effect of growth preparations on alkaline phosphatase of the tibia. Endocrinology, *45:* 129.

MATHIES, J. C., O. H. GAEBLER AND L. PALM 1949b The effect of growth hormone on hepatic and renal acid and alkaline phosphatases. Ibid., *45:* 480.

NAJJAR, V. A. 1941 The fluorometric determination of riboflavin in urine and other biological fluids. J. Biol. Chem., *141:* 355.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. Ibid., *109:* 657.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. Ibid., *147:* 399.

VAN SLYKE, D. D., AND G. E. CULLEN 1914 A permanent preparation of urease, and its use for rapid and accurate determination of urea. J. Am. Med. Assn., *62:* 1558.

YOUNG, E. G., AND C. F. CONWAY 1942 On estimation of allantoin by Rimini-Schryver reaction. J. Biol. Chem., *142:* 839.

OBSERVATIONS ON THE PROLONGED FEEDING TO RATS OF THE FLOUR MATURING AGENT, AMMONIUM PERSULFATE [1]

AARON ARNOLD AND FRANS C. GOBLE
Sterling-Winthrop Research Institute, Rensselaer, N. Y.

TWO FIGURES

(Received for publication February 24, 1950)

Ammonium persulfate has been widely used abroad for treating freshly milled flour. The amounts used are small, being of the order of one part for 10,000 parts of flour, and it is known that the persulfate ion decomposes rapidly to innocuous sulfate in the dough stage of bread making (Kent-Jones and Amos, '47; Auerbach et al., '49). In view of the unforeseen and untoward effects observed in dogs fed flour matured with nitrogen trichloride (Mellanby, '46), there is great interest in the effects of all agents proposed for this purpose. As was brought out in a recent review (Arnold, '49a), many laboratories, including this one, have examined various agents as to their suitability for producing in flour the desired maturing without giving rise to toxic effects of any sort.

The purpose of the present communication is to report the results of feeding trials with rats fed diets which contained ammonium persulfate in amounts considerably above those which would be encountered in the feeding of flour containing the usual commercial levels of this maturing agent. Included also are feeding trials with diets which contained high levels of bread baked from flour treated with ammonium persulfate.

[1] Presented in part before the American Institute of Nutrition, Detroit, April 22, 1949 (Arnold, A., and F. C. Goble, 1949, Fed. Proc., *8:* 377).

EXPERIMENTAL

Diets

The diets fed the rats had the following percentage composition: flour or dried bread, 78; lactalbumin, 10; hydrogenated vegetable oil,[2] 8; U.S.P. salt mixture no. 2, 3.5; and liver concentrate powder (1:20), 0.5. The following water-soluble vitamins were added to the diets (given in milligrams per 100 gm of diet): thiamine hydrochloride 1, riboflavin 2, niacinamide 2, pyridoxine hydrochloride 1, calcium pantothenate 4, folic acid 0.1, inositol 10, and choline chloride 100. In addition, each rat received weekly 0.1 ml of oil which supplied approximately the following: vitamin A 2,000 U.S.P. units, vitamin D 400 U.S.P. units, α-tocopherol 10 mg, and menadione 0.1 mg. Food and water were before the animals at all times.

Flour samples

Untreated and unenriched standard patent grade flour was used in the formulation of the diets.

Bread samples

The bread was baked according to the following commercial formula (given in lb.): flour 100, water 64, corn sugar 4, milk solids 2, shortening 2, yeast 2, yeast food 6 ounces, and calcium propionate 1 ounce.

It may be instructive to indicate briefly the effect on the finished loaf of ammonium persulfate additions to the flour before baking. While a 0.02% addition of persulfate to flour was more than the usual commercial level, the corresponding bread was satisfactory from the standpoint of loaf volume and cell texture. The bread made from flour to which 0.1% ammonium persulfate had been added was slightly smaller in loaf volume than the bread made from flour which contained the 0.02% persulfate. This indicated that the flour had been

[2] Primex.

overtreated and that the gas-retaining power of the dough had been lowered. However, the product baked from flour which contained 1.0% persulfate resembled bread only distantly. The dough had obviously not expanded during fermentation and baking, so that the resultant baked product closely resembled in size the unbaked dough.

To preserve and to prepare them for use in the diets, all the breads were dried overnight at approximately 60°C. (140°F.) under vacuum in a steam-heated oven. This yielded a dry product showing no evidence of toasting or charring. The dried bread was ground in a feed mill to a powder or small granules before being mixed with the rest of the dietary ingredients.

Ammonium persulfate

Pure crystalline ammonium persulfate was milled with 10% tricalcium phosphate. The resultant powder (200 mesh) could be readily dispersed throughout the flour-containing diets. It was added to the flour at the time the diets were mixed, in the following amounts: none, 0.02%, 0.1%, and 1.0%. Similar amounts of ammonium persulfate were added to the flour from which the bread was baked. For this reason, ammonium persulfate was not added to the bread-containing diets, in contrast to the flour-containing diets described above.

Rats

The studies were begun with groups of 10 female and 4 male rats. Each group was fed one of the 8 diets described above. After three weeks of feeding in the case of the flour-containing diets and one week in the case of the bread-containing diets, the rats were mated, 5 females and two males to a cage. The females were with the males until they became pregnant. The females were not bred a second time after having cast a litter.

The aforementioned first generation rats were permitted to raise to weaning size 6 of each litter. At weaning, the

litters were reduced to three rats, one male and two female young, wherever possible.

The second generation litters were each kept separately and were allowed to breed so as to produce third generation young. The resultant litters were similarly reduced to 6 and kept until weaning, at which time they were weighed, observed for unusual symptoms, if any, and discarded.

The second generation female rats were bred a second time to test further the effect of the diets on the reproductive capacity of the rats.

Hematological examinations

The blood elements of the first generation rats were examined routinely, since they were exposed to ammonium persulfate for the longest period of time. In addition, the second generation rats were examined at autopsy. The following determinations were made: hemoglobin, calculated from oxyhemoglobin readings on the photoelectric colorimeter according to the procedure recommended for the Klett-Summerson instrument, using 4% ammonium hydroxide as the diluent; total red blood cell and total white blood cell counts, using special pipettes which allow for dilutions of 1:500 and 1:20 respectively; differential white cell count, following the procedure of Coffin ('45), the values being reported in absolute numbers rather than percentages.

Post mortem examinations

About half (54 in all, 6 to 7 rats for each of the 8 groups) of the first generation rats were sacrificed after 6 months of test. The remainder (47 first generation and 74 second generation rats relatively evenly divided among the groups) were sacrificed after one year of test, the conditions of the test being considered sufficiently rigorous for valid conclusions after that period of time. The following tissues and organs were examined: heart, lungs, liver, spleen, kidneys, adrenals, pancreas, and small intestine.

RESULTS

Weight records of first generation rats

The average weight curves, together with the ranges within the groups of the first generation rats fed the flour- or bread-containing diets, are given graphically in figure 1. Comparison of the control group with the test groups fed

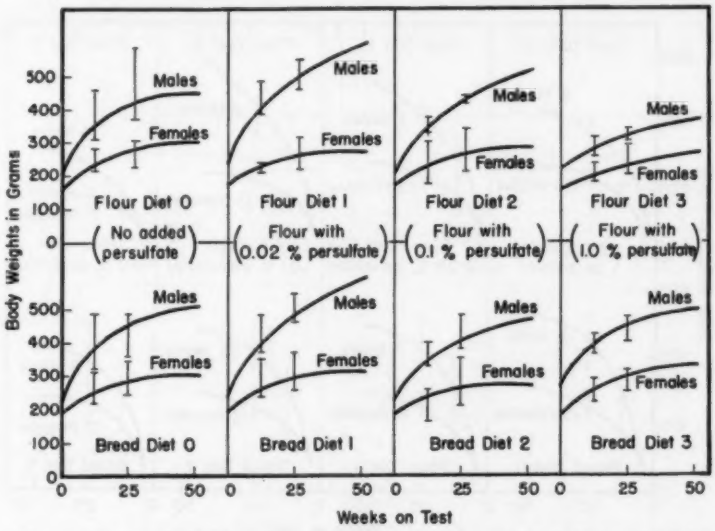


Fig. 1 Average weight curves of first generation rats fed diets containing flour, or bread baked from flour, treated with various levels of ammonium persulfate. Each group consisted of 4 males and 10 females. The ranges of the individual weights within groups are indicated by the vertical bars.

the flour-containing diets indicates that the group fed the highest level of dietary persulfate gained less weight over the experimental period of 52 weeks than did the groups fed the intermediate levels of dietary persulfate. Outwardly, the flour diet 3 animals appeared normal, however. Alternately, comparison of the control group with the test groups fed the bread-containing diets indicated no differences in weight gains among the groups.

Weight records of second generation rats

The average weight curves, together with an indication of the ranges within the groups of the second generation rats fed the flour- and bread-containing diets, are shown in figure 2. As in the case of the first generation animals, the group fed the flour-containing diet with the highest level of

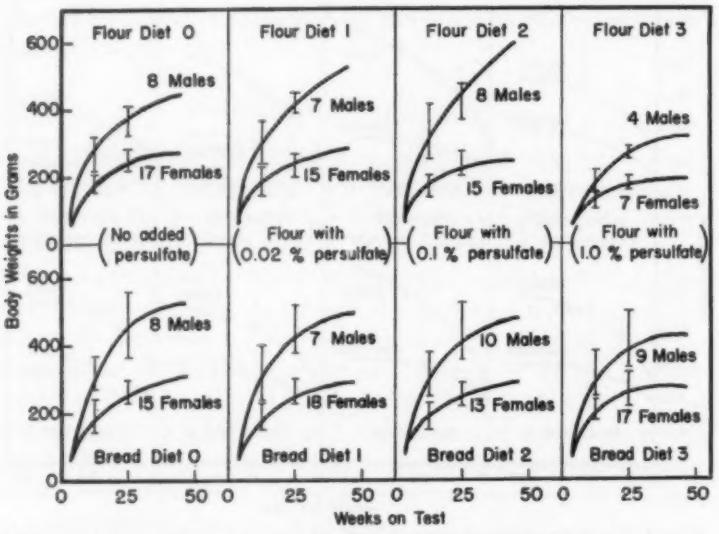


Fig. 2 . Average growth curves of second generation rats fed diets containing flour, or bread baked from flour, treated with various levels of ammonium persulfate. The ranges of the individual weights within groups are indicated by the vertical bars.

dietary persulfate gained appreciably less than the control group and the groups fed the diets with the intermediate persulfate levels. The poor condition of these animals was further evidenced by their unkempt appearance and loss of hair in irregular patches. On the contrary, the 4 groups fed the bread-containing diets were outwardly normal in appearance. The weight curves of the latter groups were quite similar

and no evidence of marked differences was apparent whether they were compared among themselves or with the weight curves of the first generation animals.

Reproduction and rearing records of the first and second generation rats

The reproduction and rearing performances of the first and second generation rats are summarized in table 1. The

TABLE 1

Summary of reproduction and rearing records of first and second generation rats

DIETARY PERSULFATE LEVEL	FIRST GENERATION RATS [1]		SECOND GENERATION RATS [2]			
			First breeding		Second breeding	
	No. of litters per no. of females bred	No. of litters weaned per no. of litters cast	No. of litters per no. of females bred	No. of litters weaned per no. of litters cast	No. of litters per no. of females bred	No. of litters weaned per no. of litters cast
%						
Flour diets						
None	9/10	9/9	12/17	5/12	7/11	2/7
0.02	8/10	8/8	12/16	10/12	3/9	0/3
0.10	10/10	8/10	12/15	9/12	7/8	5/7
1.0	7/10	4/7	4/7	2/4	0/2	...
Bread diets						
None	10/10	9/10	14/14	8/14	5/6	3/5
0.02	9/10	9/9	11/18	7/11	11/11	7/11
0.10	10/10	10/10	9/13	4/9	5/6	4/5
1.0	10/10	10/10	8/17	6/8	8/9	3/8

[1] Each group consisted of 10 females and 4 males.

[2] At weaning the litters of second generation rats were reduced, where possible, to two females and one male. The litters cast by the second generation rats were kept until weaning and were then discarded.

results obtained from the breeding of the first generation rats are consistent with the growth records given in figure 1. Seven of the 8 groups on test had excellent reproduction and rearing records. The group fed the flour-containing diet with the highest level of dietary persulfate experienced difficulty in raising the young cast and only 4 poor litters of the 7 cast were weaned.

The second generation rats, however, did not reproduce or rear litters with success equal to that of the first generation. This was indicated through two breeding trials. Examination of the results, however, does not disclose trends which relate poorer performances to an increase in persulfate level. The rats fed the flour-containing diets with 0.1% persulfate reproduced and reared their young consistently better than the flour-fed control group. The rats fed the flour-containing diets with the highest level of persulfate did not reproduce satisfactorily, as might have been anticipated from the growth

TABLE 2

Weaning weights of third generation rats

DIETARY PERSULFATE LEVEL	NUMBER OF YOUNG	AVERAGE WEANING AGE	AVERAGE WEANING WEIGHT
%		*days*	*gm.*
Flour diets			
None	31	21	41
0.02	63	25	48
0.10	52	26	54
1.0	7	35	33
Bread diets			
None	31	26	59
0.02	67	25	64
0.10	24	26	66
1.0	34	25	59

results above. The records of the second generation rats fed the bread-containing diets do not differ consistently among the groups. This is an indication that even at levels of persulfate (1.0%) which interfere seriously with fermentation and baking, no adverse effect of the bread can be detected, even in prolonged feeding trials.

Number and weaning weights of third generation rats

The data on the third generation young from the first breeding are summarized in table 2. The results confirm the trend

of those given above, in that no differences are apparent among the first three groups fed the flour-containing diets or among the 4 groups fed the bread-containing diets. The second generation rats fed the flour-containing diet with 1.0% persulfate had difficulty in rearing their young; the latter, in turn, did not reach normal weaning weights even after a prolonged rearing period.

The records of the young from the second breeding are incomplete, in that the weaning weights and times of most of the young weaned were inadvertently not entered into our records, and therefore cannot be given here.

Hematological observations

The hematological data obtained on the rats are too voluminous to present in detail. As an indication of the results obtained, the data obtained at the first reading (taken at 8 to 10 weeks on the test diet) and those obtained at the last reading (after 52 weeks of test) are given in table 3. It may be seen that there is a greater variation within groups than between groups for all categories. This demonstrates that no changes occurred which can be ascribed to persulfate ingestion in the instance of the flour-containing diets or to the ingestion of bread baked from flour which contained persulfate. The data obtained in the weeks (15 readings for most of the groups) between those given in table 3 fall in the same range as those given in detail.

The second generation rats were similarly examined before being sacrificed for histological studies. No dyscrasias or variations from the normal occurred in any of the rats receiving the bread diet with ammonium persulfate-treated flour or in any animals receiving the highest percentage of ammonium persulfate. In one of the rats receiving the 0.02% ammonium persulfate bread diet, the findings indicated a subacute myelogenous leukemia. This animal had a white blood cell count of over 488,000 (controls averaged about 16,000), of which 297,000 were myelocytes or metamyelocytes and

TABLE 3

Results of initial and final hematological examination of the first generation rats (means ± s.e.[1])

DIETARY PERSULFATE LEVEL	HEMOGLOBIN		RED BLOOD CELLS × 10^6		WHITE BLOOD CELLS × 10^3		NEUTROPHILS × 10^3		EOSINOPHILS × 10^3		LYMPHOCYTES × 10^3		MONOCYTES × 10^3	
	Wk. 10	Wk. 52	Wk. 10	Wk. 52	Wk. 10	Wk. 52	Wk. 10	Wk. 52	Wk. 10	Wk. 52	Wk. 10	Wk. 52	Wk. 10	Wk. 52
%	*mg %*													
Flour diets														
None	14.2 ± 0.2	14.2 ± 0.3	8.8 ± 0.2	9.9 ± 0.5	18.7 ± 2.1	24.8 ± 7.6	4.1 ± 0.6	7.6 ± 2.6	0.4 ± 0.1	0.4 ± 0.2	14.0 ± 5.2	17.0 ± 5.1	0.4 ± 0.1	0.3 ± 0.2
0.02		13.7 ± 0.2		8.5 ± 0.5		28.6 ± 5.4		3.7 ± 0.9		0.5 ± 0.2		24.2 ± 0.5		0.2 ± 0.1
0.10	15.5 ± 0.3	14.2 ± 0.2	8.7 ± 0.3	9.6 ± 0.6	17.9 ± 1.1	24.8 ± 3.6	4.2 ± 0.5	8.0 ± 1.8	0.5 ± 0.1	0.7 ± 0.2	12.9 ± 1.1	16.2 ± 2.2	0.4 ± 0.1	0.1 ± 0.1
1.0	15.3 ± 0.2	15.0 ± 0.3	9.5 ± 0.3	10.6 ± 0.5	16.5 ± 1.6	21.7 ± 4.2	5.1 ± 0.9	5.6 ± 1.1	0.6 ± 0.1	0.3 ± 0.1	10.7 ± 0.9	15.5 ± 3.1	0.3 ± 0.1	0.5 ± 0.3
Bread diets														
None	13.4 ± 0.5	13.4 ± 0.4	8.0 ± 0.4	8.6 ± 0.2	15.3 ± 1.5	18.2 ± 5.9	3.5 ± 0.4	3.7 ± 1.0	0.4 ± 0.1	0.3 ± 0.2	11.4 ± 1.3	14.6 ± 5.0	0.3 ± 0.1	0.1 ± 0.1
0.02		13.6 ± 0.3		9.2 ± 0.6		18.3 ± 5.5		3.3 ± 0.9		0.2 ± 0.1		14.8 ± 4.6		0.2 ± 0.1
0.10[2]	13.7 ± 0.5	13.5 ± 0.6	9.6 ± 0.6	9.1 ± 0.6	28.3 ± 8.3	19.3 ± 2.8	5.6 ± 1.4	4.8 ± 0.9	0.6 ± 0.2	0.5 ± 0.2	21.7 ± 2.1	14.0 ± 2.4	0.6 ± 0.2	0.2 ± 0.1
1.0	13.5 ± 0.4	13.6 ± 0.8	7.8 ± 0.2	9.5 ± 1.4	19.8 ± 2.8	25.0 ± 5.3	3.8 ± 0.4	7.3 ± 2.8	0.3 ± 0.1	0.6 ± 0.2	15.4 ± 2.5	17.1 ± 3.3	0.4 ± 0.1	0.2 ± 0.1

[1] Standard error.

[2] The bread 2 group was first read at week 29 rather than as indicated.

150,000 were neutrophilic band cells and incompletely segmented cells. This animal was not sacrificed at the time the others were killed but was kept under observation. Two weeks later the white cell count was approximately the same, with some tendency to more maturity of the neutrophilic series. The animal died 6 days later and at autopsy no gross abnormalities were observed. Myelogenous leukemia is a rare and interesting occurrence in the rat and its appearance in this one animal is considered to be incidental and not attributable to the conditions of the experiment.

The results based on hematological examination are thus negative, in that no significant deviations from the normal were observed.

Pathological examinations

On post mortem examination none of the rats showed any gross abnormalities. Microscopic examination of sections of the organs revealed no tissue changes attributable to the ingestion of flour-containing ammonium persulfate. No conditions were found which did not also occur in the controls except in the one rat with myelogenous leukemia, which showed the expected myelocytic hyperplasia of bone marrow and other changes secondary to this granulopoietic disorder. It is of interest to note that the gonads, in both sexes, of the rats receiving the highest percentage of ammonium persulfate in the flour diet were normal, even though this group had a poor breeding record.

DISCUSSION

From the results given above it may be noted that the rats did not exhibit any adverse effects when fed bread baked from flour treated with 0.02, 0.1, or 1% ammonium persulfate. These tests reflect the effects of the ingestion of food after the maturing agent had exerted its action, since the persulfate is known to disappear rapidly at the dough stage. The facts that the rats were fed the bread at levels considerably above those ordinarily consumed and also that rats consume more

food per unit of body weight than do larger species give added assurance of its innocuousness for larger animals. Studies with dogs fed flour treated with ammonium persulfate and steamed before feeding have led to similar conclusions (Bentley et al., '48; Arnold, '49b).

Data are presented on the chronic toxicity of ammonium persulfate obtained from diets which contained flour with 0.02, 0.1, or 1.0% of the agent. Since the usual amount of persulfate used in flour is 0.01% (Doty and Sherwood, '49), the experimental concentrations were twice, 10 times and 100 times, respectively, that likely to be encountered under practical conditions. Adverse reactions were not noted at the two lower persulfate levels, but growth and reproduction studies indicated that the diet with the hundredfold level of persulfate caused slower growth and impaired the breeding and rearing of young. Since this was in contrast to the results obtained in the bread feeding trials, and since flour consumed in appreciable amounts would come into contact with moisture at one stage or another in its preparation, thus leading to breakdown of the persulfate, it is evident that there is little likelihood of persulfate's being deleterious to the consumer when used in the treatment of flour.

Thus we are led to conclude that ammonium persulfate is an innocuous ingredient when used for maturing flour, even at levels substantially above those which could be used commercially.

SUMMARY

Flour treated with ammonium persulfate and bread baked from flour treated with ammonium persulfate were fed to rats. The treatment levels were 0.02, 0.1, and 1.0% by weight of the flour used, whereas more than 0.01% persulfate is seldom used in commercial practice. The growth of the rats and their reproduction records into three generations were followed. Additional observations included periodic hematological examinations and histological sectioning of representa-

tive tissues at the termination of the studies, after one year of medication.

The rats fed diets containing bread baked from ammonium persulfate-treated flour were not different in any observable respect from the animals fed the control bread-containing diet.

In the case of the rats fed the flour-containing diets, no differences among the rats fed the control diet and the rats fed the diets containing flour with 0.02 and 0.1% ammonium persulfate were detected. However, the rats fed the diet which contained flour with 1.0% ammonium persulfate (100 to 150 times commercial maturing levels) were definitely affected adversely. This was evident in the gross appearance of the second generation young, in the retarded growth rates of all the rats, and in the poor reproduction performances. However, hematological examination and examination of tissue sections at autopsy did not reveal the cause of the ill effects observed.

Since bread made with persulfate in amounts far in excess of those needed for maturing produced no untoward effects upon rats, since dry flour containing 10 to 15 times the normal amount of persulfate caused no ill effects upon rats, and since flour consumed in significant amounts is ordinarily moistened in some way before ingestion, thus changing ammonium persulfate to ammonium sulfate, it is concluded that ammonium persulfate may be used with safety at the levels ordinarily required for the commercial treatment of flour.

ACKNOWLEDGMENTS

The technical assistance in these studies of John C. Eggers, Christine Eddison, Giles D. Clark, Jr., and William F. Wuerdeman is hereby gratefully acknowledged. In addition, we wish to express our appreciation to Dr. Lloyd C. Miller for his assistance in the preparation of the manuscript.

The bread used in these studies was supplied through the courtesy of Earl F. Weisbrod.

LITERATURE CITED

ARNOLD, A. 1949a Current status of flour maturing agents from the biological standpoint. Trans. Am. Assn. Cereal Chem., *7:* 73.

———— 1949b Effect on dogs of flours treated with various improving agents. Cereal Chem., *26:* 46.

AUERBACH, M. E., H. W. ECKERT AND E. ANGELL 1949 Determination of persulfate in flour and dough. Ibid., *26:* 490.

BENTLEY, H. R., R. G. BOOTH, E. N. GREER, J. G. HEATHCOTE, J. B. HUTCHINSON AND T. MORAN 1948 Action of nitrogen trichloride on proteins. Production of toxic derivatives. Nature, *161:* 126.

COFFIN, D. L. 1945 Manual of Veterinary Clinical Pathology. Comstock Publishing Co., Inc., Ithaca, N. Y.

DOTY, J. M.. AND R. C. SHERWOOD 1949 Experiments with ammonium persulfate as a flour maturing agent. 34th Ann. Mtg., Am. Assn. Cereal Chem., p. 23.

KENT-JONES, D. W., AND A. J. AMOS 1947 Modern Cereal Chemistry. The Northern Publishing Co., Ltd., Liverpool, 651 pp.

MELLANBY, E. 1946 Diet and canine hysteria. Experimental production by treated flour. Brit. Med. J., *2:* 885.

THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

VI. THE INTERRELATION OF LINOLEATE AND LINOLENATE IN SUPPLYING THE ESSENTIAL FATTY ACID REQUIREMENT IN THE RAT [1, 2]

SAMUEL M. GREENBERG, CLARENCE E. CALBERT, EVELYN E. SAVAGE AND HARRY J. DEUEL, JR.

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

FOUR FIGURES

(Received for publication March 1, 1950)

In an earlier report from this laboratory (Deuel et al., '50) it was noted that greater growth obtains in rats on diets containing 10% of cottonseed oil than in animals on a fat-low basal diet supplemented daily with 20 mg of methyl linoleate. Moreover, after growth had practically ceased on this level of methyl linoleate, it was not further augmented by an increase in the daily dosage to 60 mg. When the rats were subsequently transferred to a 10% fat diet, a prompt growth response of considerable magnitude followed. Since the linoleate-supplemented rats did not reach the body weight attained by a positive control group which had received the

[1] This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Professor Anton J. Carlson of the University of Chicago, Professor Arthur W. Thomas of Columbia University, and Dr. H. W. Vahlteich and Dr. Daniel Melnick of The Best Foods, Inc., during the course of the experiments.

Contribution no. 254 from the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

[2] Presented at the meetings of the American Institute of Nutrition in Atlantic City, April 1950.

10% fat diet throughout the test, it was suggested that fat might contribute to growth in some way other than as a source of the essential unsaturated acids. This hypothesis was strengthened by the finding of the augmentative effect of a fat diet on the growth of rats previously receiving a supposedly optimum dosage of methyl linoleate.

Burr ('42) states that no absolute value can be given for the amount of linoleic acid which should be considered optimum. A very definite growth response can be noted when dosages of linoleate as low as 5 mg are given daily, while a marked effect on growth results from 10 or 15 mg amounts of this acid (Burr et al., '40; Hume et al., '40). However, Turpeinen ('38) has reported the optimum level of linoleate as being 100 mg per day. Burr and co-workers ('40) and Hume et al. ('38, '40) give the figures of 40 and 42 mg, respectively, as the linoleate requirement. On the other hand, Martin ('39) has found that 20 mg of linoleate give optimum growth, and MacKenzie et al. ('39) also obtained satisfactory growth and reproduction when the essential acid was fed at that level. Although the term "essential fatty acids" is used, Burr ('42) considers that only one is essential. Some differences in the effects of the several acids have been noted.

The present experiments were undertaken to throw further light upon the optimum requirement for linoleate in the rat. The tests were also designed to answer the question of whether the superior growth-promoting action of the cottonseed oil diet over that of a diet supplemented with linoleate may be ascribed to the fact that essential acids other than linoleic may also specifically contribute to growth, either by direct action or by a synergistic effect with linoleic acid.

METHODS

The procedures followed in the present tests were similar to those employed in our earlier work (Deuel and co-workers, '50). Weanling male and female rats of the U.S.C. strain were depleted by feeding them a fat-deficient diet until no further growth was observed. This required an average of

about 11 weeks. When the body weights of the animals had ceased to gain, they were assigned to the experimental groups, with the litters distributed as equally as possible. The rats were selected for the groups so that the average initial weights in the various categories were approximately the same.

The animals were continued on the same basal diet as was used in the depletion period throughout the remainder of the experiment except for the positive control groups, which

TABLE 1

The composition of the diets employed in the experiments

DIETARY COMPONENT	DIET 69a FAT-LOW	DIET 70a CONTROL DIET
Casein, vitamin-test [1]	20	27.8
Sucrose	71.67	31.74
Cottonseed oil [2]	0	30.0
Cellulose [3]	4	5.0
Salt mixture [4]	4	5.0
Water-soluble vitamin mixture [5]	0.33	0.46

[1] General Biochemicals, Inc.

[2] Wesson oil.

[3] Cellu flour obtained from the Chicago Dietetic Supply House.

[4] Osborne-Mendel salt mixture.

[5] The water-soluble vitamin mixture had the following percentage composition: choline and inositol, each 36.69; para-aminobenzoic acid, 18.35; thiamine chloride hydrochloride, 2.20; calcium pantothenate, 2.06; nicotinic acid, 1.83; riboflavin and pyridoxine hydrochloride, each 0.83; folic acid, 0.31; 2-methylnaphthoquinone, 0.15; and biotin, 0.06. The biotin was kindly furnished by Hoffmann-La Roche, Inc., and the folic acid by Lederle Laboratories.

received a diet containing 30% of fat from weaning or from depletion. The composition of the several diets is given in table 1.

The dosages of vitamins A and D [3] were doubled in the present diets over those used earlier (Deuel et al., '50), while the amount of α-tocopherol fed was quadrupled. The fat level of the diet fed to the positive control groups was 30% instead of the 10% in the earlier tests.

[3] Nopco preparation kindly supplied to us by Dr. B. H. Ershoff.

Each group of rats consisted of 5 males and 5 females. They were fed supplements of the substances to be tested biweekly. The total volume of each three and one-half-day dose was adjusted to 0.1 ml with ethyl laurate [4] with the exception of group 5, where the total dose fed amounted to

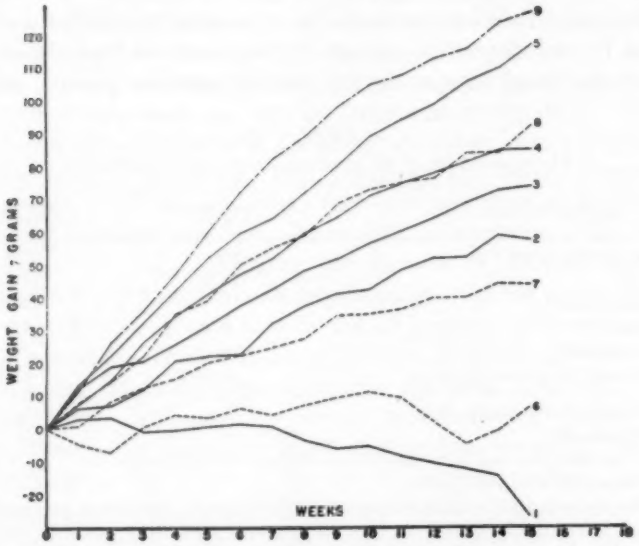


Fig. 1 Average gains in weight of groups of male rats, previously depleted of fat for 11 weeks, when supplemented over a 15-week period with methyl linoleate (2,5 mg; 3,10 mg; 4,20 mg; 5,50 mg daily), with methyl linolenate (6,10 mg daily), or with equal doses of methyl linoleate and methyl linolenate (7,5 mg each; 8,10 mg each). The negative control group (1) received no fat except ethyl laurate, while the positive control group (9) received the 30% fat diet.

0.4 ml. The supplements were given orally, using a 0.25 ml tuberculin syringe with a blunted needle for all groups. The following groups were used: 1, negative controls (ethyl laurate only); 2, 3, 4 and 5, methyl linoleate in doses of 5, 10, 20, and 50 mg, respectively, per day; 6, 10 mg of methyl

[4] Eastman.

linolenate daily; 7, 5 mg each of methyl linoleate and methyl linolenate per day; 8, 10 mg each of methyl linoleate and methyl linolenate daily; and 9, positive controls (30% fat diet) and 0.1 ml ethyl laurate. A second positive control group (10) received the high-fat diet from weaning (minus ethyl laurate) without any depletion period having intervened.

The rats were kept in individual false-bottomed cages. Food and water were supplied ad libitum. The methyl linoleate and methyl linolenate were purified esters.[5] In order to prove that the variations in body weight observed represented true growth, a determination of tibia length was made from x-ray photographs by a modification of the procedure described earlier (Deuel et al., '44). This involves measurement of the bone photograph on a screen with a 20-fold enlargement as determined from the actual length of a simultaneously projected centimeter scale.

RESULTS

The gains in weight of the male rats receiving the several supplements are shown in figure 1, while the corresponding data on the female rats are presented graphically in figure 2. The values for the actual body weights at the start, at the end of depletion, and at the conclusion of the tests are given in tables 2 and 3 for the male and female rats. Data are also recorded in these tables for group 10.

In order to determine whether the relationship between growth and linoleate intake is a quantitative one, the weight gains have been plotted against the log dose of the linoleate fed. Figures 3 and 4 give the results when the data for male and female rats are expressed in this manner.

Although for the sake of simplicity only the curves for 5 different periods are included in figures 3 and 4, the data are equally satisfactory in the case of the male rats for all the intervening weeks following the 4th week. The fact that all points fall on a straight line indicates that the growth

[5] Obtained from the Hormel Foundation, Austin, Minnesota.

response to linoleate is a quantitative one for daily doses in the range from 5 to 50 mg. Inasmuch as the relationship between gain in body weight and log dose of linoleate fed is clearest at 9 weeks, the standard error of the mean is calculated for that period. The results vary considerably in the

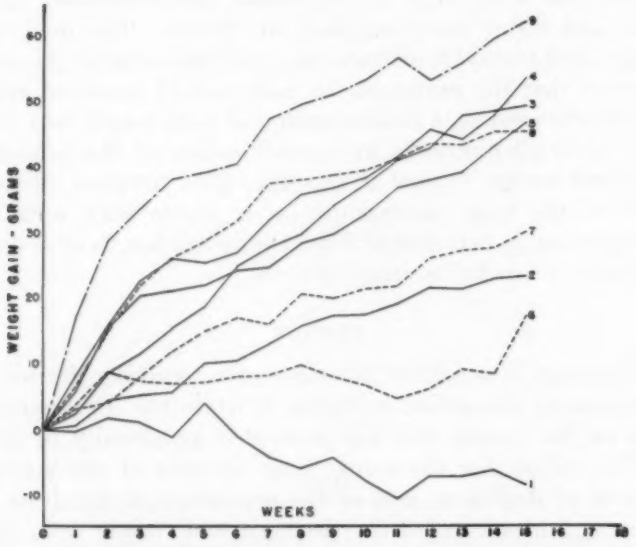


Fig. 2 Average gains in weight of groups of female rats, previously depleted of fat for 11 weeks, when supplemented over a 15-week period with methyl linoleate (2,5 mg; 3,10 mg; 4,20 mg; 5,50 mg daily), with methyl linolenate (6,10 mg daily), or with equal doses of methyl linoleate and methyl linolenate (7,5 mg each; 8,10 mg each). The negative control group (1) received no fat except ethyl laurate, while the positive control group (9) received the 30% fat diet.

female rats. As is evident from an inspection of figure 4, a straight line function obtains for the third week for doses of 5, 10, and 20 mg but not for 50 mg. This relationship is maintained through the 5th week but is no longer evident after this period, since the growth exhibited by the rats on the 20 mg dose no longer falls on the curve and approximates

TABLE 2

The body weights of male rats at weaning, after a fat depletion diet, and following supplementation with linoleate (groups 2–5), linolenate (6), equal amounts of linoleate and linolenate (7, 8), without supplement (1), or receiving a 30% fat diet throughout (10) or following depletion (9). Five rats per group

GROUP NO.	DAILY SUPPLEMENTS		BODY WEIGHT AT		GAIN IN BODY WEIGHT AT END OF FOLLOWING WEEKS OF SUPPLEMENTATION					BODY WEIGHT AT 15 WEEKS	TIBIA LENGTH [1,2]
	Methyl linoleate	Methyl linolenate	Weaning	End of depletion	3	6	9 [1]	12	15		
	mg	*mg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*mm*
1	0	0	43.6	181.8	— 0.8	1.4	— 6.2 ± 6.8	— 10.8	— 26.6	155.2	37.07 ± 0.80
2	5	0	43.4	184.0	12.0	22.8	41.4 ± 6.7	52.4	58.0	242.0	38.80 ± 0.62
3	10	0	44.6	186.6	20.8	37.6	52.0 ± 14.3	64.4	74.4	261.0	38.67 ± 0.51
4	20	0	40.6	183.6	26.8	47.6	64.8 ± 2.5	78.4	85.6	269.2	38.61 ± 0.37
5	50	0	45.4	194.6	32.6	60.0	80.4 ± 4.6	99.4	117.8	312.4	39.82 ± 0.74
6	0	10	41.2	184.8	0.4	6.2	9.6 ± 2.7	2.2	7.2	192.0	37.84 ± 0.54
7	5	5	39.2	192.2	12.6	22.6	34.8 ± 3.0	40.2	44.2	234.0	38.95 ± 0.86
8	10	10	42.0	184.0	22.2	50.2	69.0 ± 10.6	77.0	93.4	277.4	38.94 ± 0.25
9	30% fat diet after depletion; no suppl'nt.		41.8	183.0	36.0	72.4	98.4 ± 13.4	113.2	127.6	310.6	39.08 ± 0.38
10 [3]	30% fat diet throughout; no suppl'nt.		41.8	271.7	288.0 [4]	314.0 [4]	328.3 [4]	340.7 [4]	342.8 [4]	342.8	....

[1] Standard error of mean calculated by the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

[2] Determinations made at end of 18 weeks.

[3] Six rats.

[4] Body weight.

TABLE 3

The body weights of female rats at weaning, after a fat depletion diet, and following supplementation with linoleate (groups 2–5), linolenate (6), equal amounts of linoleate and linolenate (7, 8), without supplement (1), or receiving a 30% fat diet throughout (10) or following depletion (9). Five rats per group

GROUP NO.	DAILY SUPPLEMENTS		BODY WEIGHT AT		GAIN IN BODY WEIGHT AT END OF FOLLOWING WEEKS OF SUPPLEMENTATION					BODY WEIGHT AT 15 WEEKS	TIBIA LENGTH [1,2]
	Methyl linoleate	Methyl linolenate	Weaning	End of depletion	3	6	9 [1]	12	15		
	mg	*mg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*mm*
1	0	0	45.2	167.6	1.2	— 1.0	— 4.4 ± 5.2	— 7.2	— 8.4	159.2	34.77 ± 0.54
2	5	0	43.6	161.8	2.0	10.4	17.4 ± 4.0	22.0	23.4	185.2	35.32 ± 0.55
3	10	0	45.4	167.6	11.4	24.8	33.6 ± 4.7	43.0	49.4	217.0	35.78 ± 0.41
4	20	0	42.2	162.2	22.5	27.0	36.8 ± 3.2	45.8	53.8	216.0	36.00 ± 0.35
5	50	0	45.0	164.6	20.3	24.2	29.8 ± 2.6	38.0	46.8	211.4	35.84 ± 0.23
6	0	10	40.0	173.0	7.4	8.0	8.0 ± 5.0	6.2	17.2	190.2	35.62 ± 0.53
7	5	5	44.2	162.4	8.0	17.0	20.0 ± 2.4	26.2	30.2	192.6	34.94 ± 0.29
8	10	10	45.4	164.2	21.8	31.8	38.8 ± 5.7	43.6	45.5	212.8	35.86 ± 0.48
9	30% fat diet after depletion; no suppl'nt.		40.2	169.9	33.6	40.6	51.2 ± 7.5	53.4	62.4	232.2	35.96 ± 0.33
10 [3]	30% fat diet throughout; no suppl'nt.		44.7	200.7	215.7 [4]	223.4 [4]	231.2 [4]	233.0 [4]	234.2 [4]	234.2	....

[1] Standard error of mean calculated by the formula $\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

[2] Determinations made at end of 18 weeks.

[3] Six rats.

[4] Body weight.

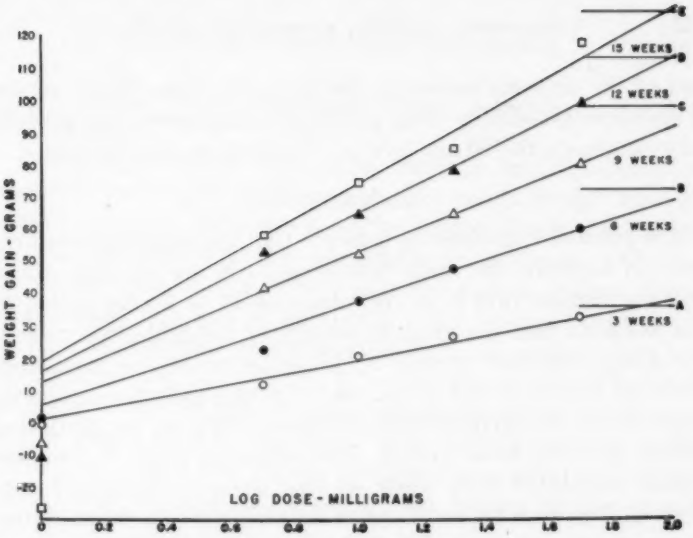


Fig. 3 The gain in weight of previously depleted male rats plotted against the log dose of methyl linoleate fed for 3, 6, 9, 12, and 15 weeks. Points A, B, C, D, and E represent the weight gains of rats on a 30% fat diet for corresponding periods, which can be used for determining the optimum linoleate requirement by extrapolation.

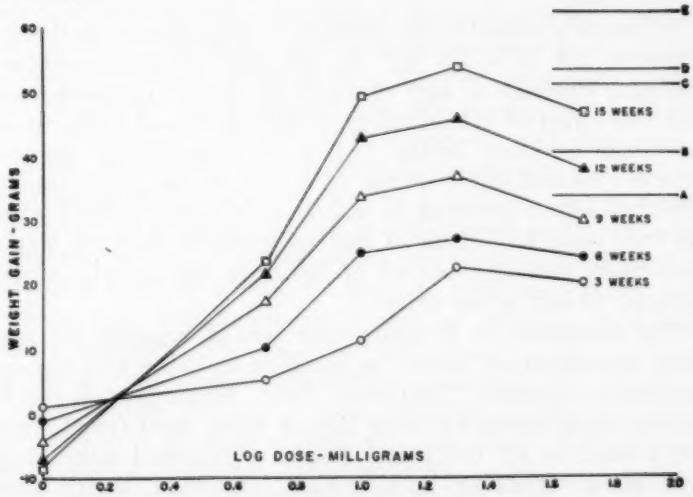


Fig. 4 The gain in weight of previously depleted female rats plotted against the log dose of methyl linoleate fed for 3, 6, 9, 12, and 15 weeks.

that of the animals receiving the 10 mg dosage. There seems to be some possibility that an actual suppression in growth may obtain on the 50 mg level of linoleate in female rats.

DISCUSSION

The present experiments indicate that the optimum requirement of linoleate for male rats exceeds 50 mg per day, while that for female rats is in the range of 10 to 20 mg daily. If one assumes that the greater growth of the male rats receiving a high-fat diet (group 9) is an index of the maximum effect of the essential acids, one can calculate the optimum requirement by extrapolation of these values on the projected weight gain-log dose curves. The optimum level of linoleate intake calculated each week on this basis is extremely constant and gives a value of approximately 100 mg daily. However, one cannot state definitely that the latter figure is the correct one until it has been demonstrated that identical growth can be produced with the 30% fat diet and with the fat-low basal diet supplemented with 100 mg of methyl linoleate daily.

Although a considerably greater growth obtains in the females on the 30% fat diet as compared with the animals receiving linoleate at any of the levels tested, it would seem that this effect of fat definitely cannot be traced solely to an increased linoleate intake. Since no appreciably greater growth obtained at the 20 mg level than at the 10 mg intake, while an actual lowering in weight gain was observed when the rats were fed 50 mg of methyl linoleate daily, it would seem that the requirement of linoleate for growth varies between 10 and 20 mg daily.

The variations in linoleate requirement reported by previous investigators cannot be ascribed to the sex of the experimental animals. Turpeinen ('38), who reported the linoleate requirement as being 100 mg daily, used female rats, while Burr et al. ('40) and Martin ('39) used animals of both sexes. However, upon critical inspection, Martin's results could be interpreted as indicating that a higher re-

quirement for the essential acids obtains in males as compared with females.

There is no ready explanation for the sex differences in essential fatty acid requirement. However, if the linoleate requirement can be related to the intermediary metabolism of fat, one might expect such differences to occur. It is well known that the fasting ketonuria in the human subject (Deuel and Gulick, '32) and in rats with fatty livers (Deuel et al., '37), as well as the exogenous ketonuria in rats and guinea pigs (Butts and Deuel, '33), is a function of sex. The levels of excretion of the ketone bodies are much higher in all cases in the female than in the male. This variation is apparently related to the more rapid disappearance of glycogen and the greater proportion of fat in the tissues of the female rat (Deuel et al., '34).

Linolenic acid is markedly inferior to linoleic acid both in growth-promoting activity and in its curative effect on the dermatitis of fat-deficiency disease. These results are at variance with those of Burr and co-workers ('40), who reported that linolenic acid was actually superior to linoleic acid in its antidermatitis action, and equal to it in its ability to produce growth. Burr et al. ('32) had previously found that the growth-promoting effects of linoleic and linolenic acids are additive. Martin ('39) disagrees with this view.

In the present tests, when methyl linolenate was fed as the only unsaturated fatty acid at a level of 10 mg daily, practically no growth occurred in either male or female rats. However, when 10 mg each of methyl linoleate and methyl linolenate were fed simultaneously, the resulting growth was identical with that noted for the group receiving 20 mg of methyl linoleate. The fact that no growth is obtained on the 10 mg level of linolenate although this acid may function in growth when linoleate is concomitantly present, indicates that each acid plays a specific role. It would thus appear that linolenic acid must be sparked by linoleic acid before it can function in growth. If this condition is met, the linolenate has the same biological potency as linoleic acid.

The present tests demonstrate that female rats on high-fat diets attain greater weights than occur when optimum amounts of methyl linoleate are available. This is in confirmation of our earlier work (Deuel et al., '50). On the other hand, the increase in body weight obtained with male rats on a 50 mg dose of methyl linoleate approached that found in animals which received the 30% fat diet. It cannot be determined on the basis of the present experiments whether the growth response of the males to 100 mg of methyl linoleate and to the 30% fat diet would be identical. It should be noted that in our earlier tests (Deuel et al., '50) the superior growth-promoting effect of the 10% fat diet as compared with the linoleate-supplemented rations was much more pronounced in the series of female rats than in the experiments on males.

The reason for the beneficial effect of the high-fat diet cannot be stated. The possibility that it may have been caused by the higher intake of α-tocopherol by the fat-fed group than by the rats on the fat-low diet would seem to be improbable, since the same variations obtained in the present series in which the α-tocopherol intake was increased 4 times over that in the earlier series. Similarly, the higher levels of vitamins A and D employed in the present series would seem to preclude the possibility that variations in these components were responsible for the above differences.

The present experiments also suggest that male rats may be especially suited for the quantitative bioassay of the essential fatty acids. In using a diet containing large amounts of fat, an appropriate control experiment would be needed in which rats were fed the same fat minus the essential acids. Studies on the possible use of such tests for quantitative measurements of essential fatty acids are now in progress.

SUMMARY

A sex difference in the requirement for linoleic acid has been found. The optimum dosage for male rats exceeds 50 mg daily, while that for female rats has been found to be between

10 and 20 mg per day. The requirement for male rats based on extrapolation data would appear to be 100 mg daily.

Linolenic acid had only slight growth-promoting action when fed alone to fat-depleted rats at the level indicated. However, when fed with suboptimum doses of linoleic acid, the resultant activity of the additional linolenate equalled that of linoleate. It is suggested that a sparking action on the part of linoleate is required before linolenate can play its role as an essential acid.

Greater growth was found in the female rats on 30% cottonseed oil diets than could be obtained on a fat-low regime where the animals were supplemented with an optimum quantity of methyl linoleate. In the case of male rats, it would appear probable that the growth of animals on a fat-free regime might be raised to that of rats on a 30% fat diet if a sufficiently high amount of methyl linoleate were administered.

The growth response of the male rats plotted against the log dose was a straight line function when linoleate in the range of 5 to 50 mg was administered. The possibility of using such a procedure for assay of the biopotency of substances containing essential fatty acids is discussed.

LITERATURE CITED

BURR, G. O. 1942 Significance of the essential fatty acids. Fed. Proc., *1:* 224.

BURR, G. O., J. B. BROWN, J. P. KASS AND W. O. LUNDBERG 1940 Comparative curative values of the unsaturated fatty acids in fat deficiency. Proc. Soc. Exp. Biol. Med., *44:* 242.

BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acids essential in nutrition. III. J. Biol. Chem., *97:* 1.

BUTTS, J. S., AND H. J. DEUEL, JR. 1933 The sexual variation in carbohydrate metabolism. II. The metabolism of diacetic acid in fasting rats and guinea pigs. Ibid., *100:* 415.

DEUEL, H. J., JR., S. M. GREENBERG, C. M. CALBERT, E. E. SAVAGE AND T. FUKUI 1950 The effect of fat level of the diet on general nutrition. V. The relationship of linoleic acid requirement to optimum fat level in the diet. J. Nutrition, *40:* 351.

DEUEL, H. J., JR., AND M. GULICK 1932 Studies on ketosis. I. The sexual variation in starvation ketosis. J. Biol. Chem., *96:* 25.

DEUEL, H. J., JR., M. GULICK, C. F. GRUNEWALD AND C. H. CUTLER 1934 The sexual variation in carbohydrate metabolism. III. The comparative glycogen and fat content of the liver and muscles of rats and guinea pigs. Ibid., *104:* 519.

DEUEL, H. J., JR., L. F. HALLMAN AND S. MURRAY 1937 Studies on ketosis. XI. The relation of fatty livers to fasting ketonuria in the rat. Ibid., *119:* 257.

DEUEL, H. J., JR., E. MOVITT, L. F. HALLMAN AND F. MATTSON 1944 Studies on the comparative nutritive value of fats. I. Growth rate and the efficiency of conversion of various diets to tissue. J. Nutrition, *27:* 107.

HUME, E. M., L. C. A. NUNN, I. SMEDLEY-MACLEAN AND H. H. SMITH 1938 Studies of the essential unsaturated fatty acids in their relation to the fat-deficiency disease of rats. Biochem. J., *32:* 2162.

———— 1940 Fat deficiency disease of rats. The relative curative potencies of methyl linoleate and methyl arachidonate, with a note on the action of the methyl esters of the fatty acids from cod liver oil. Ibid., *34:* 879.

MACKENZIE, G. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low-fat diet. Ibid., *33:* 935.

MARTIN, G. J. 1939 Studies of fat-free diets. J. Nutrition, *17:* 127.

TURPEINEN, O. 1938 Further studies on the unsaturated fatty acids essential in nutrition. Ibid., *15:* 351.

A QUANTITATIVE ESTIMATION OF THE EFFECT OF RUTIN ON THE BIOLOGICAL POTENCY OF VITAMIN C[1]

EARLE W. CRAMPTON AND LEWIS E. LLOYD
Department of Nutrition, Macdonald College, McGill University, Province of Quebec, Canada

ONE FIGURE

(Received for publication February 13, 1950)

INTRODUCTION

The nature and importance of vitamin P-active materials have been reported extensively in the literature since the middle of the last decade. The early findings were varied and sometimes contradictory, and as a result the concept of the function and mode of action of these materials was at first obscure. However, their role has become considerably clarified and recent work has brought forth a theory as to the mode of action of the many substances possessing such activity. That capillary walls become weakened under certain conditions has been known for some time. The decreased strength of the capillaries leads to hemorrhage, either through rupture of the walls or because of increased permeability. Prior to 1936, the belief existed that weakened capillaries were due wholly to a deficiency of vitamin C. In fact, capillary fragility was considered to be a part of the scurvy syndrome.

In 1936, Szent-Gyorgyi (Bentsath et al., '36) isolated a substance from both lemons and fresh paprika peppers that would restore weakened capillaries to normal. This effect was not noted when ascorbic acid alone was administered.

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Que., Canada. Journal Series no. 262.

Szent-Gyorgyi called his substance "citrin," and the active principle thereof became known as vitamin P. Bruckner and Szent-Gyorgyi ('36) stated that the extract was composed of the flavonones hesperidin and eriodictin. When the biological activity of these compounds was tested by Bentsath et al. ('37), both were found to be active; while a related compound, a flavonol named quercitrin, was inactive.

In England, the work of Scarborough is of considerable significance. Scarborough ('40) stated that vitamin P did not control in any way the clinical symptoms of gross scurvy, but that it did produce an increased capillary strength in the scorbutic subject. He found that the capillary strength of such subjects was not controlled by the administration of ascorbic acid, or of vitamins A, B_1 or D. He (Scarborough, '41) has also thrown doubt on the finding of Bruckner and Szent-Gyorgyi that citrin consists of a mixture of hesperidin and eriodictin. Taking an increase in capillary resistance in man as a criterion of vitamin P activity, he found that both hesperidin and its aglycone, hesperitin, were active. However, they can only be given orally because of their relative insolubility in an aqueous solution of suitable pH. Their activity, classed as "moderate," was greatly exceeded by that of a preparation obtained from orange peel, according to the Bentsath et al. ('36) method. Scarborough therefore stated that this latter preparation contained at least two substances which have not as yet been accurately identified. St. Rusznyak and Benko ('41) confirmed Scarborough's work by feeding a scorbutogenic diet to rats and observing the beneficial effect of citrin on the resultant weakened capillaries.

As a result of these findings, a distinct P-avitaminosis has been established, it being invariably associated with a much decreased capillary strength; it may be characterized by the development of spontaneous petechial hemorrhages, especially in areas exposed to stress. Thus, any material capable of increasing the strength of the capillary walls is said to have vitamin P activity. The chemical nature of many active extracts is still unknown but vitamin P activity has been at-

tributed variously to flavones, flavonones, flavonols and to many of their derivations. One of these, rutin, has become prominent in the literature because of its commercial availability. Griffith, Couch and Lindauer ('44) fed rutin to 14 patients with hypertension, all of whom subsequently showed increased strength in the capillary walls. This work was confirmed by Shanno ('46). Ambrose and DeEds ('47) administered rutin to rabbits and found that it decreased cutaneous capillary permeability.

The mode of action of vitamin P substances as antioxidants is stressed in the literature. The French workers Parrot et al. ('44), using epi-catechin, have postulated that vitamin P exerts its various antihemorrhagic effects by inhibiting the oxidative destruction of circulation epinephrine. This hormone (epinephrine) then appears to decrease capillary fragility, capillary permeability and bleeding time through a vasoconstrictive or tonic action on precapillary blood vessels. Somogyi ('45) added vitamin C-free extracts of lemon juice filtrate residues, known to have a high vitamin P content, to solutions of crystalline synthetic ascorbic acid. He showed that this addition served to protect against the *in vitro* oxidation of vitamin C by the ascorbic acid oxidase of white cabbage and other recognized oxidants of ascorbic acid, such as copper, the polyphenol oxidase of apples, and the peroxidase of horseradish. Richardson et al. ('47) have shown that flavonols, for example quercetin, quercitrin and rutin, are effective antioxidants for milk and lard. Furthermore, Wilson, Mortarotti and DeEds ('47) showed that rutin prolongs the action of epinephrine on intestinal strips, the prolongation presumably being due, they believe, to a protection of the epinephrine from oxidative destruction.

The concept that the protection of epinephrine from oxidative destruction is the sole mode of action of vitamin P-active materials was not accepted by Clark and Geissman ('49). Using two methods, these workers examined some 70 compounds to study the relationship between their reported vitamin P activity and their ability to potentiate the effects of

epinephrine. One method determined the extent to which those substances enhanced the effects of epinephrine on isolated intestinal segments, while the second estimated spectrographically the protection they afforded epinephrine from copper-catalyzed autoxidation *in vitro*. The authors reported that the activity series obtained by the isolated smooth muscle assay method coincided neither with that obtained by the spectrophotometric methods nor with capillary fragility-decreasing (vitamin P) activity as reported in the literature.

It is worth recalling that immediately after his isolation of citrin, Szent-Gyorgyi (Bentsath et al., '36) fed this material to guinea pigs on a scorbutogenic diet, and stated that the life of these animals was not only prolonged but that there was as well a marked decrease in the intensity of hemorrhages. He and his co-workers then suggested that experimental scurvy was caused by a combined lack of vitamins C and P. However, Zilva ('37) questioned this suggestion when he found that upon the administration of citrin the onset of scurvy was not delayed, nor was the fatal termination of the disease retarded in guinea pigs on a scorbutogenic diet. He also showed that sub-minimum prophylactic doses of ascorbic acid administered to such guinea pigs produced a pathological condition resembling that obtained by Szent-Gyorgyi and his colleagues by the administration of a daily dose of 1 mg of citrin. Zilva explained the results of the Hungarian workers by postulating that their crude extract of citrin contained traces of ascorbic acid. When Szent-Gyorgyi et al. failed to reproduce their earlier findings, the suggested role of vitamin P as a modifying factor in the scurvy syndrome was abandoned.

Recent work, however, indicates that there is a relationship between vitamin P-active materials and ascorbic acid. Cotereau et al. ('48) showed that catechin, when fed to guinea pigs, permitted a higher than average tissue storage of ascorbic acid, which was not the result when the latter was supplied alone, even at a level of 10 mg per day. Of more significance is the work of Ambrose and DeEds ('49), in which it was

found that in guinea pigs receiving sub-minimum amounts of ascorbic acid, ascorbic acid plus rutin, and ascorbic acid plus quercetin, the post mortem findings were essentially the same, except that the last two groups showed fewer fresh hemorrhages than the animals receiving ascorbic acid alone. They also stated that the combined supplements of ascorbic acid and rutin apparently prolonged the life of scorbutic guinea pigs.

The results of both groups of workers may be feasibly accounted for by the assumption that the two vitamin P-like materials were protecting the ascorbic acid from oxidative destruction. The work of Ambrose and DeEds shows the modifying effect of vitamin P-active materials on the scurvy syndrome; not directly, as originally assumed by Szent-Gyorgyi, but indirectly through their sparing action on ascorbic acid. Thus, Szent-Gyorgyi's earlier work would be explained if his crude extract did contain traces of ascorbic acid, as suggested by Zilva; while Zilva's findings would also be valid, since he fed purified hesperidin and eriodictin, with no vitamin C present.

EXPERIMENTAL

The specific object of this experiment was to test the extent to which rutin affected the apparent biological potency of synthetic ascorbic acid and of vitamin C from a natural source (commercial orange-grapefruit juice).

The odontoblast method of bioassay was used and each animal was maintained on test for a 42-day period. As a basis for dosing, the orange-grapefruit juice was analyzed chemically for ascorbic acid, using both the dichlorophenol indophenol method of Hochberg, Melnick and Oser ('43) and the dinitrophenylhydrazine method of Roe and Oesterling ('44).

Healthy 28-day-old guinea pigs, weighing approximately 300 gm at the beginning of the assay period, were fed syn-

thetic ascorbic acid or orange-grapefruit juice as the sole source of vitamin C. Rutin was fed to half the animals on each of these treatments.

Design of the assay

The general design of this assay is shown in the allotment plan set forth in table 1. The experiment was carried out in two replicates of 64 animals each. A total of 8 guinea pigs, 4 males and 4 females on each treatment, was involved for the complete test.

TABLE 1

Allotment plan

Showing distribution of 128 animals to treatment groups (8 in each group) and mean height of odontoblast cells (microns) for each treatment

RUTIN TREATMENT	ASCORBIC ACID LEVEL	MEAN HEIGHT OF ODONTOBLASTIC CELLS WITH ASSAY MATERIALS	
		Ascorbic acid	Orange-grapefruit juice
	mg	*microns*	*microns*
None	0.50	27.1 [1]	26.4
	0.79	31.8	33.0
	1.26	37.7	37.8
	2.00	42.4	41.5
100 mg rutin	0.50	31.7	31.5
	0.79	36.1	36.5
	1.26	39.8	40.1
	2.00	41.9	42.3

[1] Averages of 4 males and 4 females in each group.

Animals

Guinea pigs from the stock colony at Macdonald College were weaned at 21 days of age, and after 7 ± 2 days from the weaning date, were randomized within sexes to each of the 16 feeding groups.

Diets

All animals received ad libitum the Macdonald College guinea pig diet no. 6, plus water, throughout the 42-day test

period. The percentage composition of this diet is as follows: oats, 15.0; wheat, 13.0; beet pulp, 20.0; linseed oilmeal, 12.5; skim milk powder, 15.0; fish meal, 5.0; brewers' dried yeast, 10.0; feeding molasses, 5.0; bone char, 4.0; and salt (0.1% KI), 0.5.

TABLE 2

Analysis of variance

SOURCE OF VARIATION	D.F.	$S(x-\bar{x})^2$	VARIANCE s^2	VARIANCE RATIO	
				Observed	Necessary minimum (P = 0.05)
Total	95	2001.95			
Sub groups	23	1950.02			
Levels	2	1505.41	752.71	990.1	3.14
Rutin	1	316.49	316.49	416.5	3.99
Sex	1	0.97	0.97	1.3	3.99
Materials	1	1.05	1.05	1.4	3.99
Levels × rutin	2	25.53	12.76	16.8	3.14
Levels × sex	2	0.69	0.35	0.5	3.14
Levels × materials	2	3.83	1.92	2.5	3.14
Rutin × sex	1	0.29	0.29	0.4	3.99
Rutin × materials	1	0.001	0.001	0.09	3.99
Sex × materials	1	0.36	0.36	0.5	3.99
Second order interactions	7	95.40	10.50	13.8	2.08
Third order interactions	2				
Calculated values	4				
Error	68	51.93	0.76		
		S.D. = 0.87			

The diet was prepared by grinding, mixing, and pressing the mixture into pellets approximately one-eighth of an inch in diameter. Previous work in this laboratory has shown that the minor variations in consumption under ad libitum feeding have no direct influence on this method of bioassay. Thus no feed records were kept.

Supplements

Vitamins A, D and E were administered weekly by a calibrated syringe. The dose was such that every animal was

given the equivalent of a daily intake of 3 mg of alpha-tocopherol, 425 I.U. of vitamin A, and 48 I.U. of vitamin D_2.

The ascorbic acid was dissolved in distilled water immediately before dosing, while a fresh tin of orange-grapefruit juice was opened daily. Both these sources of vitamin C were administered directly by calibrated syringe.

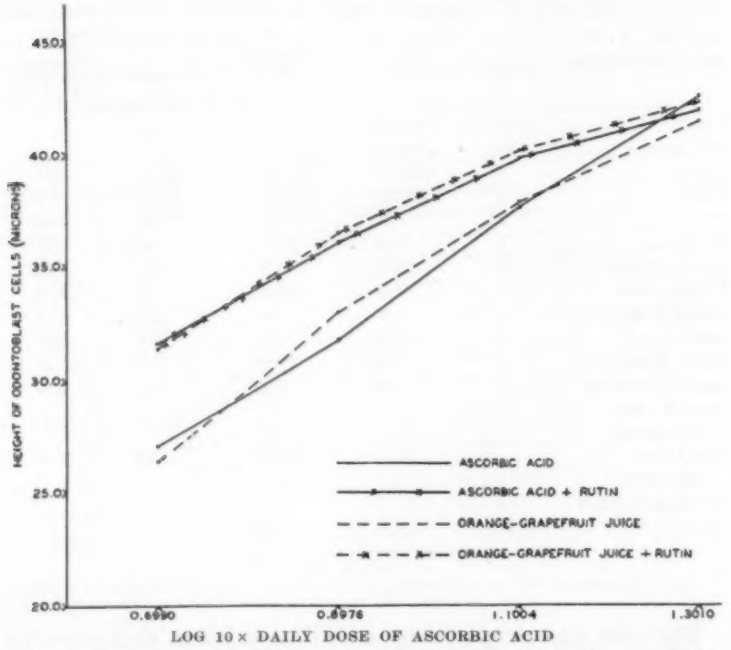


Fig. 1 Regression of height of odontoblast cells on daily intake of vitamin C from ascorbic acid and from orange-grapefruit juice, showing the effect of added rutin.

Approximately 100 mg of crystalline rutin were fed daily to each guinea pig involved. They were given orally by means of a calibrated tube.

Analysis of experimental data

At the conclusion of the 42-day feeding period, each animal was sacrificed. The incisor teeth were prepared for micro-

scopic examination of the odontoblast cells according to the procedure developed in this laboratory (Crampton, '47). The odontoblast cell measurements (microns) were analyzed statistically, with the variance partitioned as shown in table 2. The method of Bliss and Marks ('39) was applied in calculating the relative potencies of the vitamin C sources.

TABLE 3

The vitamin C potencies of ascorbic acid and orange-grapefruit juice, showing the effect of rutin

MATERIAL	RELATIVE POTENCY [1]	LIMITS OF RELATIVE POTENCY P = 0.05	BIOLOGICAL POTENCY	PROBABLE LIMITS OF BIOLOGICAL POTENCY P = 0.05	MEAN VITAMIN C POTENCY — CHEMICAL (HOCHBERG ET AL., '43)
	%	%	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Ascorbic acid	100.0	99.0–101.0	35.0	34.6–35.4	
Ascorbic acid + rutin	156.2	143.5–170.0	54.7	50.2–59.5	
Orange-grapefruit juice	101.8	97.7–106.0	35.7	34.3–37.1	35.3
Orange-grapefruit juice + rutin	152.9	141.6–165.0	53.1	49.5–57.7	35.3

[1] Differences between relative potencies greater than 1.5 percentage units are statistically significant.

RESULTS

Chemical

The average apparent vitamin C potencies, as determined by the two chemical methods on random samples of orange-grapefruit juice, were: (1) Method of Roe and Oesterling ('44), 42.9 mg/100 ml; (2) method of Hochberg et al. ('43), 35.3 mg/100 ml. The standard deviation of the individual determinations with the Roe method was $\pm$ 5.1% and with the Hochberg $\pm$ 3.5%.

Because of the smaller variability of results, figures obtained with the latter method were used in all calibrations as well as in comparisons between the biological and chemical values of vitamin C potency.

Biological

The mean heights of the odontoblast cells of the guinea pigs receiving synthetic ascorbic acid and orange-grapefruit juice, each with and without rutin and at the 4 dosage levels of 0.5 mg, 0.79 mg, 1.26 mg, and 2.00 mg, are summarized in table 1. These results are illustrated graphically in figure 1.

The inclusion of the 2.00-mg level of vitamin intake caused a deviation in the linear regression of odontoblast height on logarithm of dose, and this level was therefore excluded from the analysis of variance.

The potencies of ascorbic acid plus rutin, orange-grapefruit juice, and orange-grapefruit juice plus rutin were determined relative to synthetic ascorbic acid. Figures illustrating the effect of rutin and the values of the mean ascorbic acid potencies of the orange-grapefruit juice, as determined chemically and biologically, are given in table 3.

DISCUSSION

A very close agreement between the mean vitamin C potencies as determined biologically and chemically (Hochberg, Melnick and Oser, '43) was found for the orange-grapefruit juice. This agreement was in accordance with previous work in this laboratory.

When fed at ascorbic acid levels where it could exert its full effect, rutin produced striking results. Considering the apparent biological potency of synthetic ascorbic acid as 100%, it may be seen from table 3 that the effect of supplementing this material with rutin was to increase its apparent biological potency by 56.2%. Furthermore, when orange-grapefruit juice constituted the source of vitamin C rutin increased the apparent biological potency of the ascorbic acid therein by 48.8%.

At levels of vitamin C intake above these, rutin had progressively less effect. Presumably at these levels sufficient ascorbic acid was available to the animals and the administration of rutin had little or no effect on the height of the

odontoblast cells, which was at, or was approaching, a maximum.

These quantitative findings tend to support the concept that rutin, and other vitamin P-like substances, carry out their physiological function as antioxidants. Rutin either made more ascorbic acid available or delayed its "*in vivo*" oxidative destruction in the original source.

CONCLUSIONS

Rutin significantly increased the apparent biological value of ascorbic acid when the vitamin was supplied in sub-maximum amounts either in crystalline form or from a natural source.

The mechanism of action of rutin in this role is uncertain, but prevention of the "*in vivo*" oxidative destruction of vitamin C would explain these results.

LITERATURE CITED

AMBROSE, A. M., AND F. DEEDS 1947 Effect of rutin on permeability of cutaneous capillaries. J. Pharm. Exp. Ther., *90:* 359.

———— 1949 The value of rutin and quercetin in scurvy. J. Nutrition, *38:* 305.

BENTSATH, A., I. ST. RUSZNYAK AND A. SZENT-GYORGYI 1936 Vitamin nature of flavones. Nature, *138:* 798.

———— 1937 Vitamin P. Ibid., *139:* 326.

BLISS, C. I., AND H. P. MARKS 1939 The biological assay of insulin. Q. J. Pharm., *XII:* 82, 182.

BRUCKNER, V., AND A. SZENT-GYORGYI 1936 Chemical nature of citrin. Nature, *138:* 1057.

CLARK, W. G., AND T. A. GEISSMAN 1949 Potentiation of effects of epinephrine by flavonoid (vitamin P-like) compounds. Relation of structure to activity. J. Pharm. Exp. Ther., *95:* 363.

COTEREAU, H., M. GABE, E. GERO AND J. L. PARROT 1948 Influence of vitamin P upon the amount of ascorbic acid in the organs of the guinea pig. Nature, *161:* 557.

CRAMPTON, E. W. 1947 The growth of the odontoblast of the incisor teeth as a criterion of vitamin C intake of the guinea pig. J. Nutrition, *3:* 491.

GRIFFITH, J. Q., J. F. COUCH AND M. A. LINDAUER 1944 Effect of rutin on increased capillary fragility in man. Proc. Soc. Exp. Biol. Med., *55:* 228.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1943 Photochemical determination of reduced and total ascorbic acid. Ind. Eng. Chem., Anal. Ed., *15:* 182.

PARROT, J. L., J. LAVOLLAY AND P. GALMICHE 1944 The action of adrenalin upon the resistance of capillaries and its increase due to the influence of vitamin P (epi-catechin). C. rend. Soc. biol., *138:* 330.

RICHARDSON, G. A., M. S. EL-RAFEY AND M. L. LONG 1947 Flavones and flavone derivatives as antioxidants. J. Dairy Sci., *30:* 397.

ROE, J. H., AND M. J. OESTERLING 1944 Determination of dehydroascorbic acid and ascorbic acid in plant tissues by 2,4-dinitrophenylhydrazine method. J. Biol. Chem., *152:* 511.

SCARBOROUGH, H. 1940 Deficiency of vitamin C and vitamin P in man. Lancet (London), *2:* 644.

———— 1941 On the nature of vitamin P. Chem. and Ind., *60:* 618.

SHANNO, R. L. 1946 Rutin: A new drug for the treatment of increased capillary fragility. Am. J. Med. Sci., *211:* 539.

SOMOGYI, J. C. 1945 An investigation of substances which inhibit vitamin C degradation. Zschr. Vitaminforsch., *16:* 134.

ST. RUSZNYAK, I., AND A. BENKO 1941 Experimental vitamin P deficiency. Science, *94:* 25.

ST. RUSZNYAK, I., AND A. SZENT-GYORGYI 1936 Vitamin P: Flavonols as vitamins. Nature, *138:* 27.

WILSON, R. H., T. G. MORTAROTTI AND F. DEEDS 1947 Some pharmacological properties of rutin. J. Pharm. Exp. Ther., *90:* 120.

ZILVA, S. S. 1937 Vitamin P. Biochem. J., *31:* 915.

THE EFFECTS OF EXCESS TRYPTOPHAN AND EXCESS LYSINE ON THE PRODUCTION OF RICKETS IN THE RAT

WALDEMAR DASLER

WITH THE TECHNICAL ASSISTANCE OF ELEANOR M. CORDES

Department of Biochemistry, Chicago Medical School, Chicago, Illinois

ONE FIGURE

(Received for publication March 9, 1950)

The work upon which this report is based was stimulated by the preliminary observation of Bauer ('47) that the addition of tryptophan to a moderately rachitogenic diet appeared to retard the development of a rachitic metaphysis in the growing rat. Since this effect of supplementary tryptophan on rachitogenesis, if true, would be opposite to the effect of lysine (Francis, '47), the effects on rachitogenesis of excess DL-tryptophan and of excess L-lysine have been compared, using an objective criterion — namely, the per cent of femur ash — as a basis for comparison.

EXPERIMENTAL

One hundred and ten Holtzman-Sprague Dawley male rats, 24 days of age and weighing 44 to 54 gm, were divided into groups and fed the U.S.P. rachitogenic diet 2 supplemented with DL-tryptophan [1] and with L-lysine monohydrochloride in excess, as indicated in table 1. The supplements were incorporated into the basal ration and the rations were fed ad libitum. Ten additional rats of the same age, sex, and weight

[1] The DL-tryptophan used in this study was generously supplied by the Dow Chemical Company, Midland, Michigan.

range were killed at the start of the experiment and a femur was removed from each animal for bone ash determinations.

Ten animals from each experimental group were sacrificed at 10-day intervals and their femurs removed. The femurs from all animals were extracted continuously in a Soxhlet extractor, first for 20 hours with ethyl alcohol, then for 20 hours with ethyl ether. Following removal of all ether, the bones were dried for 18 hours at 110°C., cooled in a desicca-

TABLE 1

Effects of tryptophan and lysine added in excess to U.S.P. rachitogenic diet 2 [1]

SUPPLEMENT	NO. OF RATS	AVERAGE GROWTH PER RAT PER 10-DAY PERIOD			FEMUR ASH (mean ± standard error) after		
		0–10 days	10–20 days	20–30 days	10 days	20 days	30 days
		gm	*gm*	*gm*	%	%	%
	10	8.0			39.77 ± 0.46		
None	10	8.6	8.6			33.30 ± 0.61	
	10	9.4	8.6	7.0			27.40 ± 0.47
	10	4.8			41.08 ± 0.72		
1% DL-tryptophan	10	4.5	3.3			35.06 ± 0.65	
	10	4.0	6.6	5.8			30.26 ± 0.63
1% L-lysine monohydrochloride	10	12.4			35.02 ± 0.61		
	10	12.5	8.8			30.10 ± 0.43	
	10	12.4	7.8	7.9			28.04 ± 0.68
1% DL-tryptophan plus 1% L-lysine monohydrochloride	10	7.8	7.0			32.58 ± 0.39	
	9 [2]	9.3	7.1	5.1			28.50 ± 0.31 [2]
Normals	10	Femur ash at start of experiment: 46.38 ± 0.29.					

[1] U.S.P. rachitogenic diet 2 (Steenbock diet 2965): ground whole yellow corn 76%; ground wheat gluten, 20%; $CaCO_3$, 3%; NaCl, 1%.

[2] Since one widely divergent bone ash value in this group deviated from the mean of the other 9 by 8.0 times their standard deviation, the data obtained from this rat were not included in calculating growth and bone ash values for this group. Including the bone ash datum of this rat gives a mean and standard error of 29.25 ± 0.80 for the group.

tor, and weighed. They were ashed individually at 850°C. for one hour, cooled in a desiccator, and the ash weighed. The determination of bone ash, therefore, was carried out essentially as prescribed by the Association of Official Agricultural Chemists ('45).

RESULTS

The results are summarized in table 1. The addition of an excess of L-lysine to the rachitogenic basal ration resulted, during the first 10-day period, in a growth rate which approached a level 50% higher than that of the control group. During subsequent 10-day periods the growth rate dropped back to a level approximating that of the control animals. The addition of excess DL-tryptophan, on the other hand, caused a halving of the growth rate during the first period. A decreased rate of growth continued also during the second and third periods. When the basal diet was supplemented with both lysine and tryptophan, the growth rate was not equal to or greater than that of the lysine-supplemented rats, which might be the expected result, but instead was roughly the same as that of the control group; i.e., intermediate between the rates of growth obtained with each of the two supplements alone.

The average bone ash of the rats receiving tryptophan decreased more slowly than that of the control animals (table 1). The difference in bone ash between these two groups after 10 days was not statistically significant. As the experiment was continued, however, the bone ash values diverged (see also fig. 1) until the difference at 30 days was highly significant, having a probability of only one in 500 that the difference was due to chance alone.

The bone ash values decreased more rapidly in the rats receiving lysine than in the control group. The differences between the means of these two groups were highly significant both at 10 days and at 20 days, the probability that these differences were due to chance being less than one in 1,000 in both instances. At 30 days, however, these two groups

showed no significant differences in femur ash (cf. also fig. 1). This finding is an expected one, since, at 30 days, the bone ash values of both groups were approaching the physiological lower limit.

Rats receiving both tryptophan and lysine had an average femur ash which was not significantly different from that of

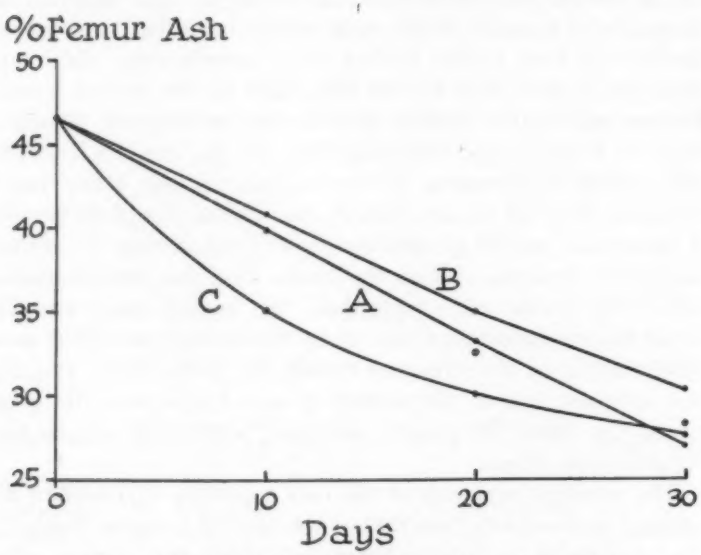


Fig. 1 Effect of amino acid supplements on femur ash.

Curve A — Control group. Rachitogenic diet only.

Curve B — Rachitogenic diet plus 1% DL-tryptophan.

Curve C — Rachitogenic diet plus 1% L-lysine monohydrochloride.

o — Rachitogenic diet plus 1% each of DL-tryptophan and L-lysine monohydrochloride.

the control animals. This was true for both the 20-day and the 30-day periods. The 10-day value was not determined. The mean bone ash at 20 days, however, differed very significantly from the means of the groups receiving either tryptophan or lysine alone, being lower for the animals receiving lysine and higher for those receiving tryptophan.

The results of line tests carried out on the distal ends of the radii and ulnae, while less clear-cut than the femur-ash results, appeared in general to confirm the latter. After the 20- and 30-day periods, an impartial observer was able to pick out the groups having received tryptophan as exhibiting, on the average, slightly less wide rachitic metaphyses than the controls.

DISCUSSION

The results of the bone ash determination are consistent with the well-known facts that an increased rate of growth during rachitogenesis accelerates the production of rickets, and that a decreased rate of growth has the opposite effect. The finding of Francis ('47) that the addition of lysine to the Steenbock diet increases the growth rate as well as the severity of the rickets was confirmed. It is interesting to note that, in this experiment, the increased rate of growth on the lysine-supplemented diet was limited to the first 10-day period. The growth rate then fell off when another nutrient became the limiting factor. One would expect, however, that after the growth stimulus supplied by the lysine had been nullified by a second deficiency, the growth rate would drop to a level below that of the control rats, the demand for the missing nutrient having been intensified by the initial spurt in growth. This was not the case.

The decreased rate of growth of the rats receiving excess tryptophan is an unexpected finding in view of the repeated observations of others that both tryptophan and niacin improve the growth of rats receiving diets containing a high level of corn (Krehl et al., '45, '46a, b; Salmon, '47). The present study is complicated, of course, by the fact that the Steenbock diet, although high in corn, is at the same time deficient in other nutrients. Another factor which may have had some effect on the results is the fact that the level of added tryptophan was 5 times the minimum estimated requirement for growth (Rose, '37) and considerably higher than that used by other workers feeding high corn diets. It was, for instance, 20 times the supplementary level used by

the Wisconsin group (Krehl et al., '45, '46a, b) and 3.3 times the level used by Salmon ('47).

Because the lysine content of the Steenbock rachitogenic diet is suboptimum (Francis, '47), the decreased rate of growth of animals receiving the tryptophan-supplemented diet might be taken to indicate an increased demand for lysine when excess tryptophan is supplied. This apparently is only partly true, since animals receiving a level of lysine which was well above their dietary requirement grew less well when tryptophan was also present in the diet in excess.[2]

SUMMARY

Rats receiving the U.S.P. rachitogenic diet 2, supplemented with 1% L-lysine monohydrochloride, exhibited an increased rate of growth for the first 10 days and decreased femur ash for the first 20 days of the experiment when compared to control animals receiving no supplement. After 30 days the per cent ash of the femurs of the lysine-fed and the control groups were the same.

DL-tryptophan, added to the rachitogenic diet at a level of 1%, gave rise to a decreased rate of growth and an increased femur ash.

The growth rate and femur ash of the group of rats receiving both supplements simultaneously did not differ significantly from those of the control animals.

ACKNOWLEDGMENT

The author is grateful to Dr. A. H. Ryan of the Department of Physiology and Pharmacology for assistance in the statistical evaluation of the data.

[2] The lysine content of the basal ration as calculated from data given by Block and Bolling ('45) = 0.14 gm (76% corn) + 0.36 gm (20% wheat gluten) = 0.50 gm/100 gm ration. The lysine in 1% lysine monohydrochloride = 0.80 gm/100 gm ration. The total lysine content of the lysine-supplemented ration = 0.80 + 0.50 = 1.3 gm/100 gm ration. The lysine requirement of the rat for growth (Rose, '37) = 1.0 gm/100 gm ration.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official and Tentative Methods of Analysis. 6th ed., p. 626, Washington, D. C.

BAUER, C. D. 1947 Personal communication.

BLOCK, R. J., AND DIANA BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill.

FRANCIS, P. S. 1947 Improvement of the Steenbock rachitogenic diet by a supplement of lysine. J. Assoc. Offic. Agr. Chem., *30:* 364.

KREHL, W. A., L. M. HENDERSON, J. DE LA HUERGA AND C. A. ELVEHJEM 1946a Relation of amino acid imbalance to niacin-tryptophan deficiency in growing rats. J. Biol. Chem., *166:* 531.

KREHL, W. A., P. S. SARMA, L. J. TEPLY AND C. A. ELVEHJEM 1946b Factors affecting the dietary niacin and tryptophan requirement of the growing rat. J. Nutrition, *31:* 85.

KREHL, W. A., L. J. TEPLY, P. S. SARMA AND C. A. ELVEHJEM 1945 Growth-retarding effect of corn in nicotinic acid-low rations and its counteraction by tryptophan. Science, *101:* 489.

ROSE, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298.

SALMON, W. D. 1947 Relation of corn products to the requirement of the rat for dietary nicotinic acid. J. Nutrition, *33:* 169.